Solutions that meet
your demands for

# food safety testing

Our measure is your success.

## Excellent choices for food applications

products | **applications** | software | services

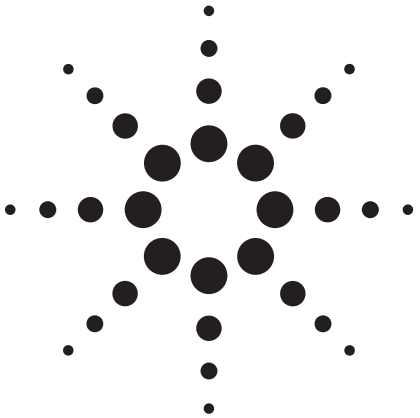
Agilent Technologies

# Productivity Tools

## Applications

> Return to Table of Contents
> Search entire document

# Retention Time Locking with the MSD Productivity ChemStation



Technical Overview

## Introduction

A retention time is the fundamental qualitative measurement of chromatography. Most peak identification is performed by comparing the retention time of the unknown peak to that of a standard. It is much easier to identify peaks and validate methods if there is no variation in the retention time of each analyte.

However, shifts in retention time occur frequently. Routine maintenance procedures such as column trimming alter retention times. In a multi-instrument laboratory running duplicate methods, the retention times for each instrument will differ from each other, even when run under nominally identical conditions. These differences in retention times mean that each instrument must have a separate calibration and integration event table, making it time-consuming to transfer methods from one instrument to another. Differences in retention time also complicate comparison of data between instruments over time.

## What Is Retention Time Locking?

Retention time locking (RTL) allows a close match of retention times on one Agilent Technologies GC/MSD or GC system to those on another like system with the same nominal column. By making an adjustment to the inlet pressure, the retention times on one system can be closely matched to those on another system using the same nominal column. The ability to very closely match retention times from one system to another can greatly reduce the time it takes to develop and transfer methods. “RTLocked” methods can also compensate for degradations in chromatographic performance. The ability to correct for degrading chromatographic performance, optimize lab resources, and still provide the correct answer saves time, money, and results in significant productivity gains.

Using GC/MS, it is also possible to screen samples for the presence of target compounds using a mass spectral database of RTL spectra. The RTL mass spectral database provides additional confirmatory information in spite of changes to the chromatographic system. One such database is the G1672AA Pesticide RTL Library. This database allows for quick and easy screening of pesticide samples.

## When Should I Lock My Methods?

Locking or relocking your methods should be done whenever you make any changes to the chromatographic system or move methods from one system to another (GC or GC/MS). To establish and maintain a locked method, RTL should be performed whenever:

- The column is changed or trimmed
- The method is installed on a new instrument
- A detector of different outlet pressure is used (GC vs. MS)
- System performance is validated
- Troubleshooting chromatographic problems

## How Does It Work?

The process of RTL is to determine what adjustment in inlet pressure is necessary to achieve the

desired match in retention times. To lock a given method for the first time or for the reasons above, you must first develop a Retention Time vs. Pressure (RT vs. P) calibration curve. Using an established method, multiple (five) injections of the standard are used to calculate the retention times at predefined inlet pressures. The use of an automatic sampler simplifies this process. The RT vs. P calibration data for each of the five injections are saved and used to correct locked methods.

The five defined pressures are:

- Target pressure –20%
- Target pressure –10%
- Target pressure (nominal method pressure)
- Target pressure +10%
- Target pressure +20%

Even when using columns with the same part number (same id, stationary phase type, phase ratio, and same nominal length), separate and different locking calibration curves may be needed. Other examples of when separate locking calibration curves are required include:

- Systems with different column outlet pressures (MSD/vacuum, FID/atmospheric)
- Columns differing from the "nominal" length by more than 15% (for example, due to trimming)
- Systems where the predicted locking pressure falls outside the range of the current calibration

### Selecting the Standard Compound for RTLocking

A specific compound (usually one found in the normal method calibration standard) must be chosen and then used for both developing the locking calibration and locking all future systems. The compound, or target peak, should be easily identifiable, symmetrical, and should elute in the most critical part of the chromatogram. Compounds that are very polar or subject to degradation should be avoided.

Once the target compound has been chosen and all other chromatographic parameters of the method have been determined, the five calibration runs are performed. The resulting RT vs. P calibration curve data are saved. The software is then used to select and integrate the peak used for locking.

## Creating an RTL Method

The following is an overview of the actual steps taken to acquire the RT vs. P calibration curve data, selecting the compound/peak to use and locking the method.

### Acquiring the RTLock Data

From **Instrument Control**, select the **Instrument** menu and **Acquire RTLock Calibration Data....** Agilent Technologies GCs are the only ones supported. See Figure 1.

If you are using an automatic sampler, the five sample injections will be made automatically. This example illustrates how RTL works on a GC/MS system. Upon completion of the five-sample analysis, Data Analysis will begin and display the nominal MS total ion chromatogram (TIC). If the system was configured as a stand-alone GC, all five chromatograms will be displayed. From these displays, you will select the compound that will be used for RTLocking of the method.

### Selecting the RTLock Compound

Use the mouse to select the compound or peak that you would like used for locking. For GC-only mode, you must select one peak from each of the five chromatograms for RTLocking. Once you have made your selection, you will be asked to allow the software to automatically find the remaining peaks. You may choose to zoom the display for better visibility. See Figure 2.

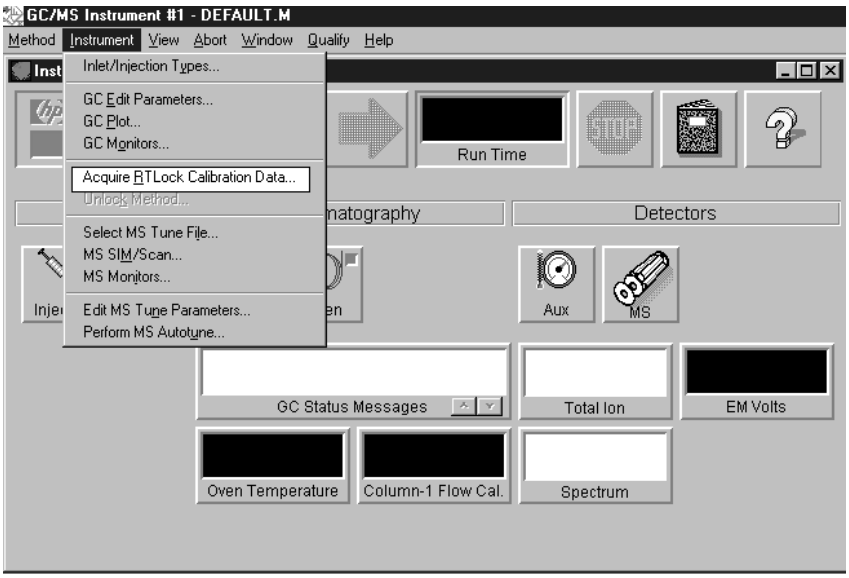



**Figure 1. From instrument control, you select the mode of data acquisition for RTL.**

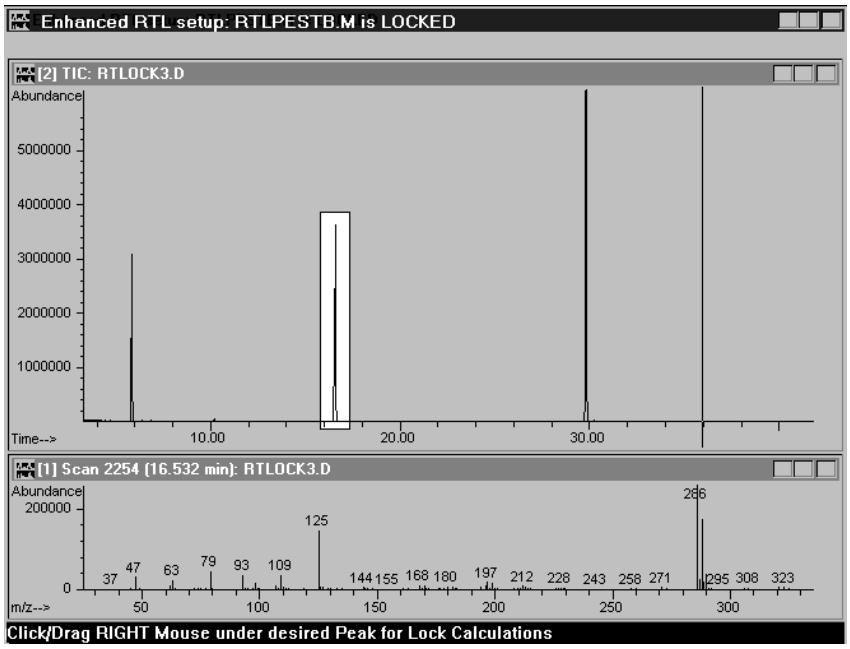



**Figure 2. From this panel, the user selects the peak used for RTL. In this example the second peak has been selected. The spectrum of the selected compound is also displayed and is used to confirm the RTL compound.**

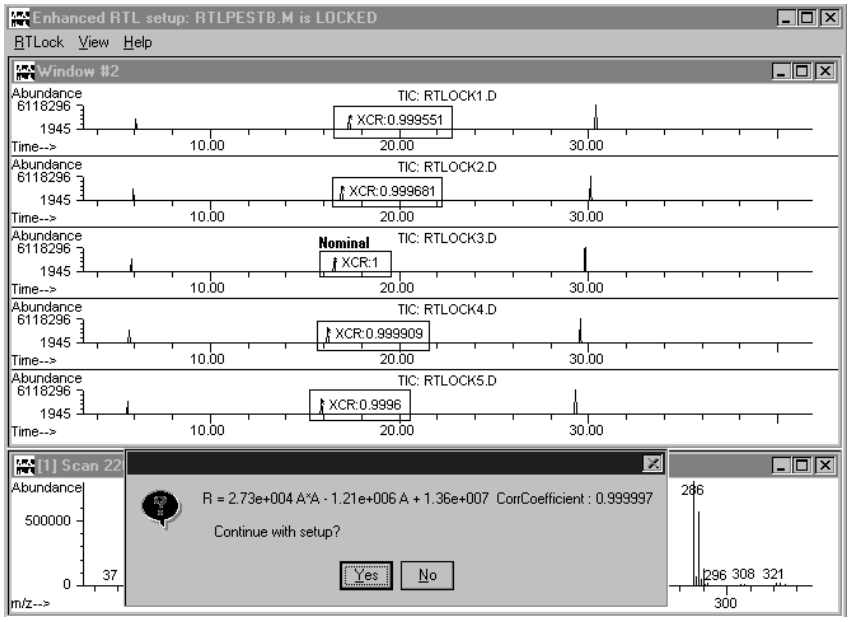



**Figure 3. When the RT vs. P calibration curve equation is calculated, the correlation coefficient is determined for the RTLocked compound in each of the five calibration samples. The resulting coefficient is displayed at each peak. The "nominal," or no change to pressure calibration sample, has a correlation of 1.**

### Calculating the RTLock Curve and Saving a Method

Once the RTLock compound has been selected, the new RT vs. P curve for each compound will be displayed. To select a new compound and calculate new RT vs. P curves, reset the nominal MSTIC from the **RTLock** menu item. Select a new compound as the RTL compound and from the **RTLock** menu item, select **Calculate New Curve from Selected Peaks**. This will generate new RT vs. P calibration curves. The new RT vs. P calibration curve equation will be displayed on the screen along with the correlation coefficient. Select **Yes** to either create a new, or update an existing, RT vs. P calibration file. See Figure 3.

Next, you can enter the name and retention time of the RTLock compound. See Figure 4 and Figure 5.

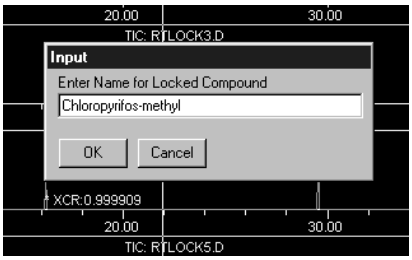



**Figure 4. Enter or confirm the name of the RTLock compound.**

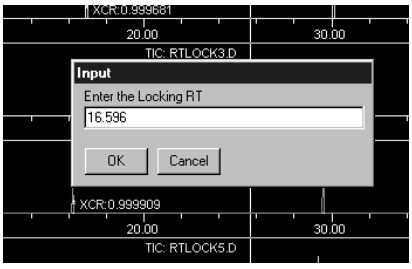



**Figure 5. Enter or confirm the retention time of the RTLock compound.**

You will then be asked to confirm the RTLock pressure that has been calculated and will be used for that method. Select **Yes** to confirm the new RTLock pressure and save the method. See Figure 6.

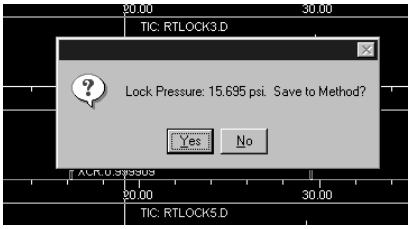



**Figure 6. By selecting Yes, you RTLock and save the method.**

### View Current RTLock Method Setpoints and Report

Once the system is calibrated and locked, you can view and confirm the RTLock setpoints by selecting **View Current Method Setpoints.** See Figure 7.

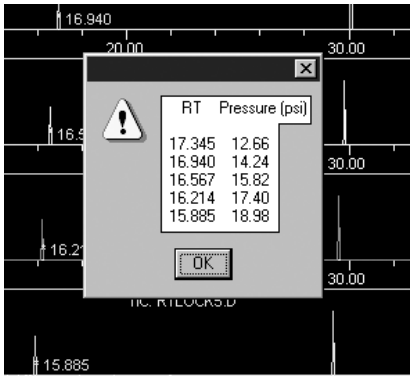



**Figure 7. Viewing the RTLock retention times and corresponding pressures for the RTLock method.**

A report is also available that provides detailed information regarding the RTLock method. See Figure 8. The report includes:

- Method name
- Calibration date
- Instrument name
- Operator name
- Status of method (on or off)
- RTLock compound name
- Tabular retention time/ pressure calibration table
- Maximum deviation
- RTLock curve equation and correlation coefficient data
- Locked retention time information (file name, acquisition date, instrument name, and operator name)
- Report date

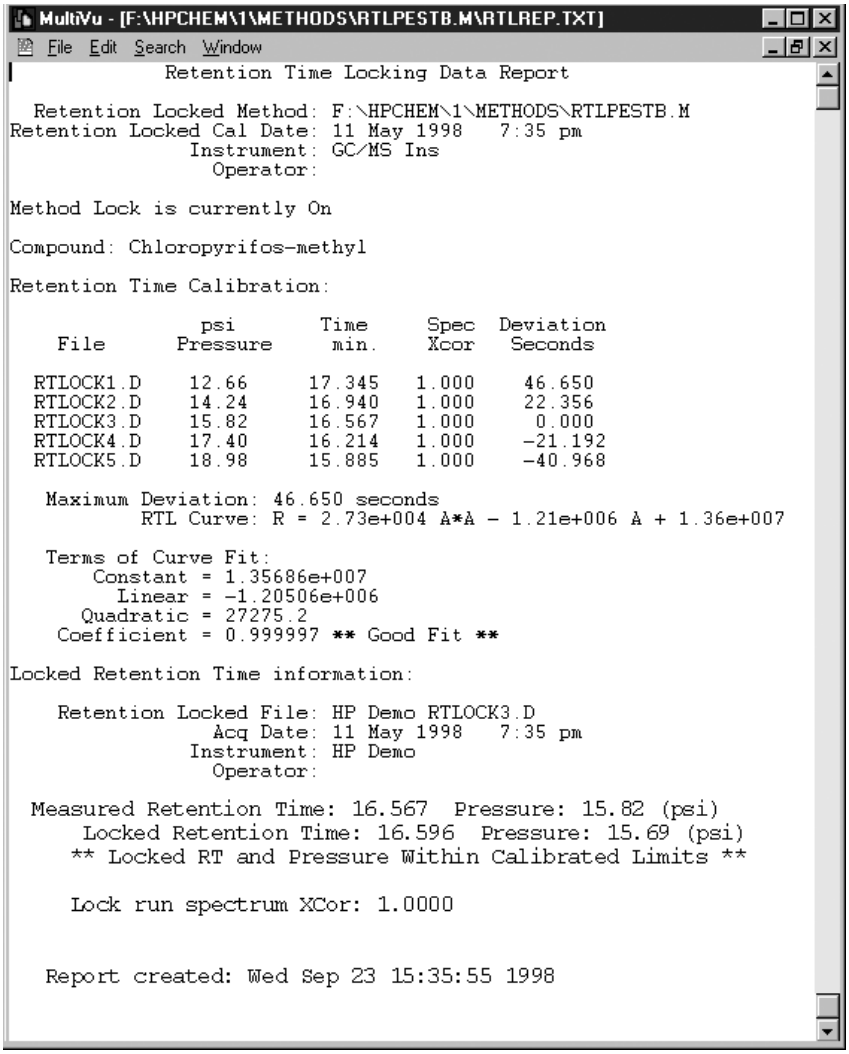

MultiVu - [F:\HPCHEM\1\METHODS\RTLPESTB.M\RTLREP.TXT]
File Edit Search Window
Retention Time Locking Data Report
Retention Locked Method: F:\HPCHEM\1\METHODS\RTLPESTB.M
Retention Locked Cal Date: 11 May 1998 7:35 pm
Instrument: GC/MS Ins
Operator:
Method Lock is currently On
Compound: Chloropyrifos-methyl
Retention Time Calibration:
File psi Pressure Time min. Spec Xcor Deviation Seconds
RTLOCK1.D 12.66 17.345 1.000 46.650
RTLOCK2.D 14.24 16.940 1.000 22.356
RTLOCK3.D 15.82 16.567 1.000 0.000
RTLOCK4.D 17.40 16.214 1.000 -21.192
RTLOCK5.D 18.98 15.885 1.000 -40.968
Maximum Deviation: 46.650 seconds
RTL Curve: R = 2.73e+004 A*A - 1.21e+006 A + 1.36e+007
Terms of Curve Fit:
Constant = 1.35686e+007
Linear = -1.20506e+006
Quadratic = 27275.2
Coefficient = 0.999997 ** Good Fit **
Locked Retention Time information:
Retention Locked File: HP Demo RTLOCK3.D
Acq Date: 11 May 1998 7:35 pm
Instrument: HP Demo
Operator:
Measured Retention Time: 16.567 Pressure: 15.82 (psi)
Locked Retention Time: 16.596 Pressure: 15.69 (psi)
** Locked RT and Pressure Within Calibrated Limits **
Lock run spectrum XCor: 1.0000
Report created: Wed Sep 23 15:35:55 1998

**Figure 8. Example RTLock Report**

### Running an RTLock Method

Once the RTL method is created, you can analyze and process new samples. This requires that you unlock and relock the methods without editing the quantitation calibration data. The following example demonstrates how retention times might change when column maintenance is performed or a method is moved to a new GC/MS system. See Figure 9. Once the method is RTLocked, new samples are analyzed and retention times corrected. See Figure 10.

### Unlocking and Relocking a Method

Once a method is locked you may unlock or relock it using the same or different compounds or after additional maintenance. To define a new compound for RTLocking, select **RTLock Setup** from the **View** menu in **Data Analysis**. See Figure 11. The nominal RTLock sample that represents the method you are working on will be displayed. Once again, use the mouse to identify a new RTLock compound.

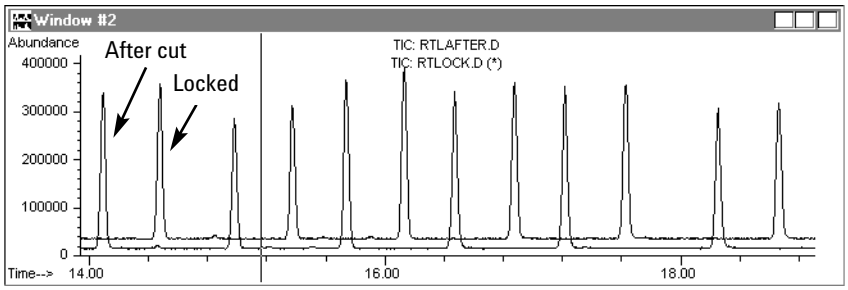



**Figure 9. Overlay of an RTLocked data file (labeled Locked) and the resulting data file after clipping one meter off the column (labeled After Cut).**

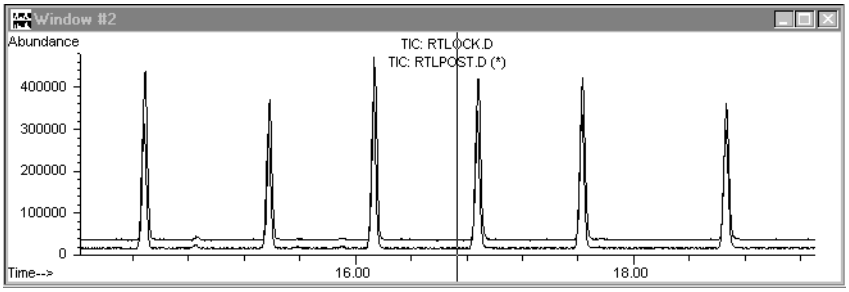



**Figure 10. Offset overlay of RTLocked data file and the RTCorrected datafile show peaks overlapped and corrected for column maintainence.**

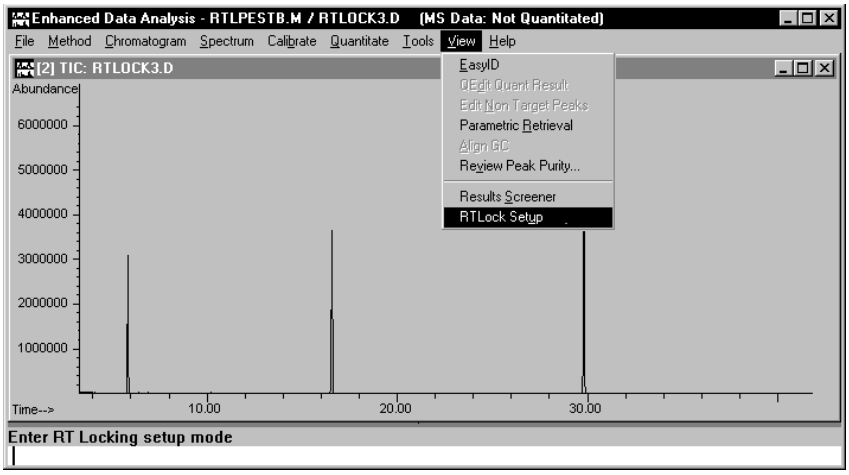



**Figure 11. To define a new compound for RTLocking, select** RTLock Setup **from the** View **menu in data analysis.**

After selecting the compound to RTLock, from the **RTLock** menu item, select **Calculate New Curve** from **Selected Peaks**. This will generate a new RT vs. P calibration curve. The new RT vs. P calibration curve equation will be displayed on the screen along with the correlation coefficient. Select **Yes** to create a new or update an existing RTLock calibration. See Figure 12. From the RTLock menu you can also:

- View current setpoints
- Calculate the RT vs. P curve
- Restore the original chromatograms
- Report the RTLock calibration
- Unlock the method
- Relock the method

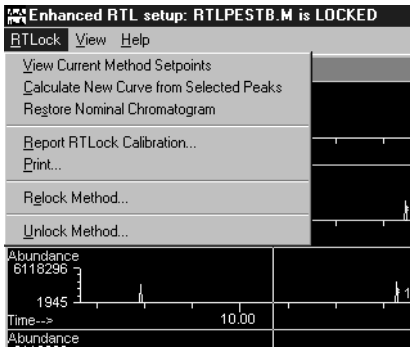



**Figure 12. RTLock view and menu choices.**

## Summary

RTLocking provides an easy and flexible tool that can be used to reduce the time and complexity often associated with routine chromatographic maintenance. It allows methods to be transferred between like GC/MS systems without time-intensive edits to the quant database and reacquisition of standards. It also simplifies the process for executing routine chromatographic maintenance. RTLocking can minimize mistakes and provide a productivity improvement for most applications by reducing the time and setpoint changes required to update a method.

## For More Information

For more information on our products and services, visit our Web site at www.agilent.com/chem.

Agilent shall not be liable for errors contained herein or for incidental or consequential damages in connection with the furnishing, performance, or use of this material.

Information, descriptions, and specifications in this publication are subject to change without notice.

© Agilent Technologies, Inc. 2008

Printed in the USA
May 5, 2008
5989-8574EN

# Low-Pressure Retention Time Locking with the 7890A GC

## Application

**HPI**


## Authors

Courtney Milner and Russell Kinghorn
BST International
41 Greenaway Street
Bulleen, VIC 3105
Australia

Matthew S. Klee
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808
USA



## Abstract

**Retention time locking was introduced over a decade ago with the Agilent 6890 gas chromatograph. The next generation of GC from Agilent – the 7890A – has enhanced and extended the functionality previously available, including a new electronic pneumatic control system capable of pressure control to the third decimal place. This application demonstrates the ability of the EPC system to be used for retention time locking at low pressures, in this case with a 320-µm column on a 5975 GC-MS.**


## Introduction

The introduction of retention time locking (RTL) with the 6890 GC gave users a new way of improving productivity by eliminating the need to constantly update compound retention time data whenever a column was trimmed or replaced. It also allowed the same methods to be run on multiple systems with the same retention times. The introduction of eMethods further enhanced the portability of these methods.

Most RTL-based methods were established on GC/MS systems, where a typical head pressure for a 30 m × 0.25 µm id column is > 10 psi. With setability to two decimal places, four-digit pressure setpoints for such columns (for example, 11.54 psig) result in excellent inter-instrument and intra-instrument RTL precision.

Amongst the many optimization tasks in GC method development is deciding on the best column dimension to select. This can realistically only be determined with full knowledge of the sample characteristics and analysis goals (that is, components of interest, complexity, detection limits required, and the matrix of the sample). Larger diameter columns have the advantages of ruggedness and sample capacity over smaller dimension columns. The larger the diameter of the column, the less pressure is needed to establish optimal flows. However, for the most precise RTL, one needs the ability to set pressure very precisely. With two-decimal-place precision at low pressures (for example, 1.28 psig), locking a system to a target retention time is less precise for column dimensions, such as 0.32-µm columns.

The 7890 GC's fifth generation EPC provides excellent low-pressure control, and with third-decimal-place control of the pressure, providing the precision demanded for RTL at low pressures. This application explores the suitability of the 7890 for RTL at low pressures with a 320-µm column and uses a translation of the method described in Agilent Technologies publication 5989-6569EN, "Reliable transfer of existing Agilent 6890/5973GC/MSD methods to the new 7890/5975 GC/MSD."

## Experimental

The method used for this example was translated using the Agilent Method Translator software to convert to a 250-µm id column method to a 320-µm method. A series of standards was run from 10 ppb to 5 ppm to illustrate the performance of the method, followed by trimming approximately 45 cm off the column and relocking the method.

**Table 1. Method Conditions**

| | |
|---|---|
| Column | HP-5 25 m × 0.32 µm × 0.52 µm<br>p/n 19091J-112 |
| Carrier gas | Helium, constant pressure mode –<br>nominal 3.761 psi |
| RTL peak | Fluoranthene at 11.112 min |
| Split/splitless inlet | 300 °C, pulsed splitless 7 psi for 0.3 in,<br>50 mL/min purge at 0.75 min |
| Oven | 55 °C (1.1 min) to 320 °C (6.5 min) at<br>22.88 °C/min |
| Sample | 1-µL injection, PAH 0.01 to 5 ppm<br>concentration range |
| MSD | Scan 45–400 u<br>Samples = $2^2$<br>Autotune EM + 200V<br>Source = 230 °C<br>Quad = 150 °C<br>Transfer line = 280 °C |

## Results and Discussion

The test sample with 16 polynuclear aromatic hydrocarbons (PAHs) was chosen for this example as it covers a wide range of physical properties and provides several challenging separations. This requires the retention time precision to be as tight as possible to ensure correct identification of the peaks of interest, and relocking must be effective or peak identification will fail on the different column.

Samples were injected in triplicate to measure the retention time precision of the locked method at different concentrations from 0.01 to 5 ppm. Table 2 shows the performance metrics for the retention time and also the correlation co-efficient for the analysis.

The initial retention times in Table 2 were achieved at a head pressure of 3.761 psig. This setpoint was determined from the retention time calibration and relocking process to be appropriate to achieve the target retention time of 11.112 min for the locking compound fluoranthene. Table 3 shows the calibration data from the RTL runs.

**Table 3. RTL Data**

| Run | Pressure (psi)* | Retention time (mins) | Deviation (seconds) |
|---|---|---|---|
| RTLOCK1.D | 3.01 | 11.212 | 6.018 |
| RTLOCK2.D | 3.38 | 11.166 | 3.264 |
| RTLOCK3.D | 3.76 | 11.112 | 0.000 |
| RTLOCK4.D | 4.14 | 11.070 | –2.508 |
| RTLOCK5.D | 4.51 | 11.020 | –5.508 |
| Maximum deviation | | 6.018 seconds | |
| Correlation co-efficient | | 0.999 | |

* Even though pressure setpoints used for RTL calibration need only be to be to two decimal places, the ability to precisely set locking pressures based on the calibration curve requires setability to the third decimal place.

Figure 1 shows the calibration curves corresponding to the linearity metrics summarized in Table 2.

**Table 2. Retention Time Precision of Low-Pressure RTL**

| | Average retention time | RSD (%) | Calibration linearity ($r^2$) | RT before relocking | Relocked RT | ΔRT when relocked |
|---|---|---|---|---|---|---|
| Naphthalene | 5.884 | 0.055 | 0.997 | 5.809 | 5.876 | 0.008 |
| Acenapthylene | 7.753 | 0.045 | 0.997 | 7.676 | 7.746 | 0.007 |
| Acenaphthene | 7.968 | 0.044 | 0.997 | 7.893 | 7.959 | 0.009 |
| Fluorene | 8.562 | 0.048 | 0.997 | 8.486 | 8.556 | 0.006 |
| Phenanthrene | 9.702 | 0.089 | 0.997 | 9.621 | 9.692 | 0.010 |
| Anthracene | 9.752 | 0.044 | 0.996 | 9.671 | 9.746 | 0.006 |
| Fluoranthene | 11.119 | 0.031 | 0.996 | 11.041 | 11.116 | 0.003 |
| Pyrene | 11.388 | 0.090 | 0.996 | 11.308 | 11.383 | 0.005 |
| Chrysene | 12.808 | 0.094 | 0.996 | 12.719 | 12.799 | 0.009 |
| Benz[a]anthracene | 12.855 | 0.046 | 0.997 | 12.774 | 12.849 | 0.006 |
| Benz[b]fluoranthene | 14.285 | 0.045 | 0.995 | 14.168 | 14.277 | 0.008 |
| Benz[k]fluoranthene | 14.314 | 0.069 | 0.995 | 14.201 | 14.306 | 0.008 |
| Benz[a]pyrene | 14.815 | 0.035 | 0.996 | 14.686 | 14.807 | 0.008 |
| Indeno[1,2,3-cd]pyrene | 17.088 | 0.051 | 0.996 | 16.890 | 17.074 | 0.014 |
| Dibenz[a,h]anthracene | 17.098 | 0.066 | 0.995 | 16.903 | 17.082 | 0.016 |
| Benzo[ghi]perylene | 17.725 | 0.089 | 0.997 | 17.508 | 17.709 | 0.016 |

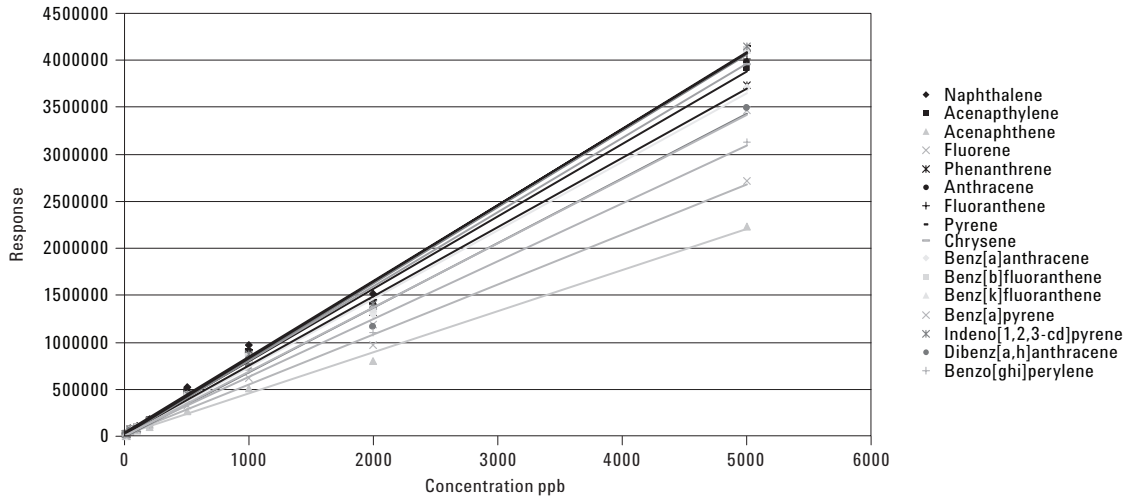



**Figure 1. GCMS calibration curves for 16 PAHs using 320-µm id column with low-head pressure.**

Figure 2 presents overlayed chromatograms for the replicate sample injections, showing the excellent precision of the replicate injections summarized in Table 2.

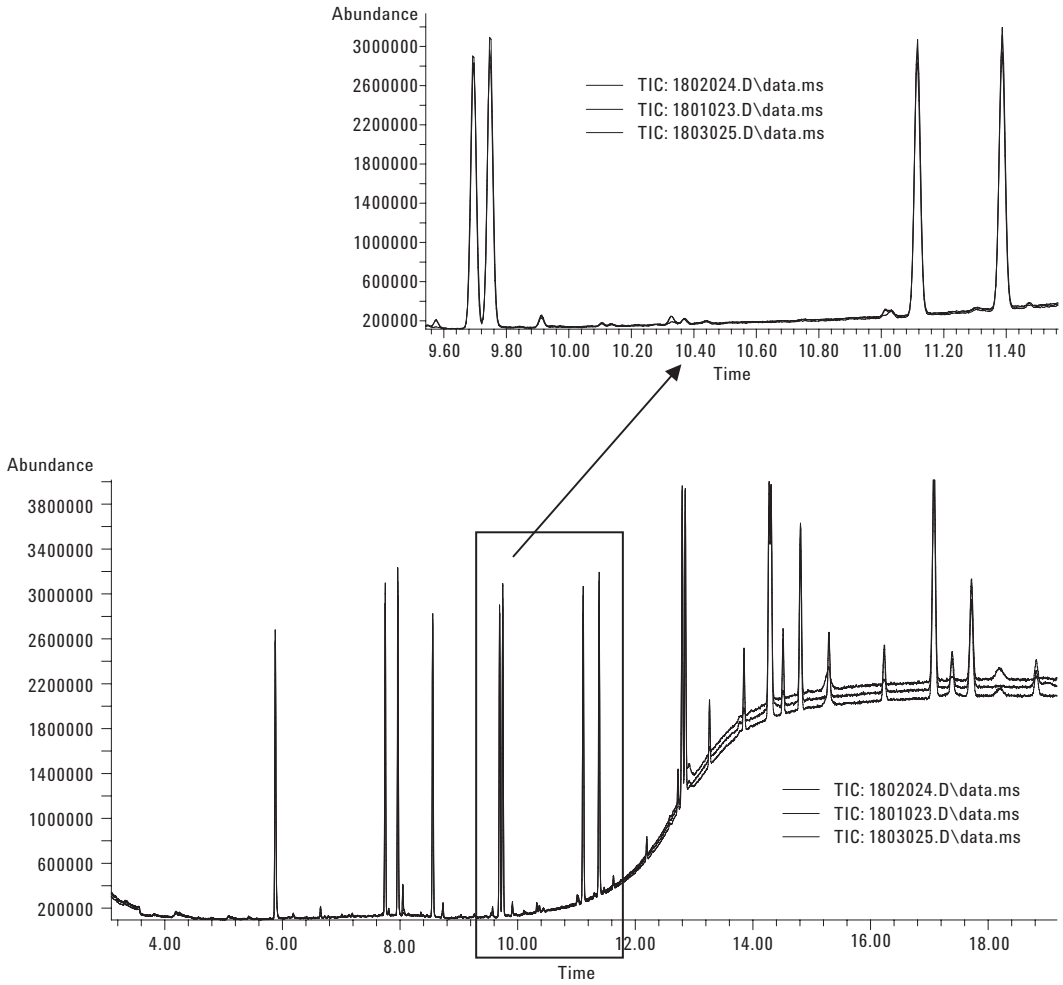



**Figure 2. Overlay of three replicate injections of 2 ppm standard prior to column maintenance and relocking, including a zoomed area of the four peaks from phenanthrene to pyrene.**

A 45-cm length of column was removed from the column to simulate typical maintenance that may be performed on a column during routine use. The method was then relocked and the sample re-run to check the efficacy of relocking at low pressure. The relocked method had a resulting pressure of 3.168 psig. The change in locking pressure can, over time, provide guidance as to the extent at which column trimming can be undertaken without the need for full re-locking of the method.

Figure 3 shows an overlay of the before and after column trimming and the extent of the retention time change. Figure 4 presents an overlay of chromatograms, one of the originals and one after column trimming and relocking. The last two columns of Table 2 compare the relocked retention times of target compounds to the originals.

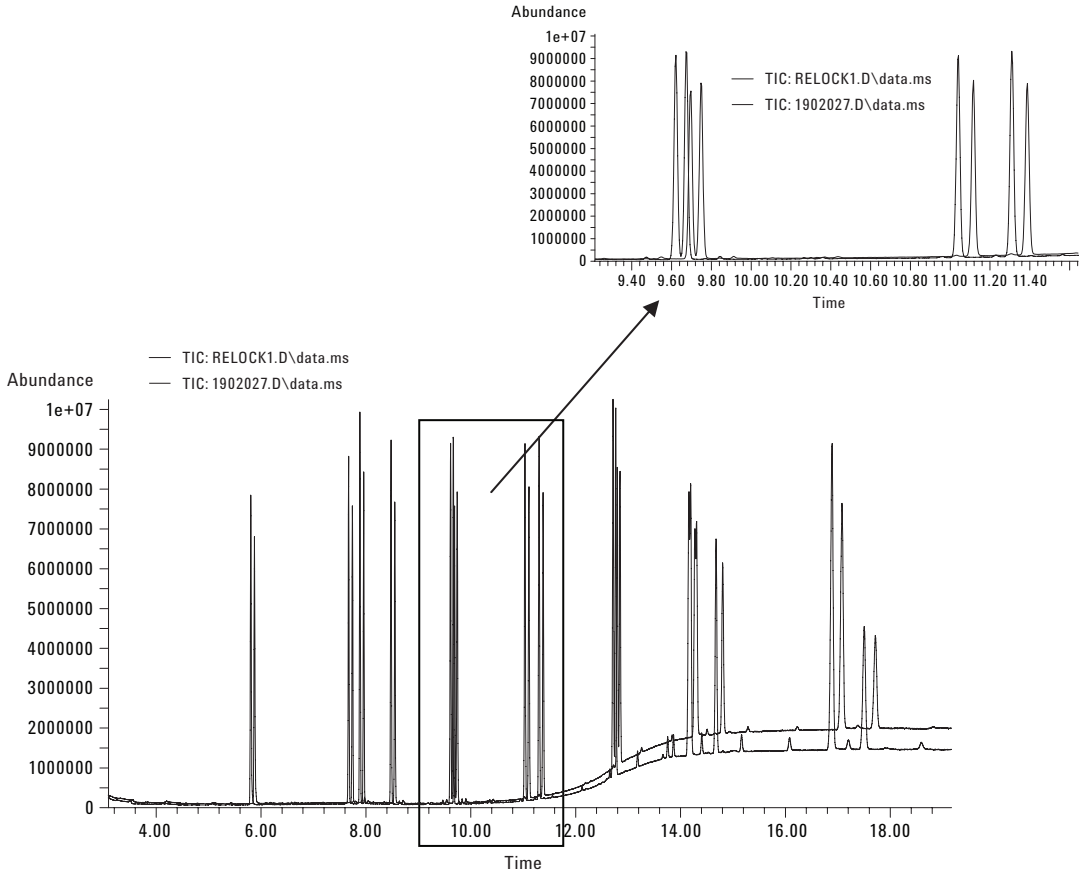



**Figure 3. Overlay of injection before and after column maintenance showing the extent of retention time variation, including a zoomed area of the four peaks from phenanthrene to pyrene**

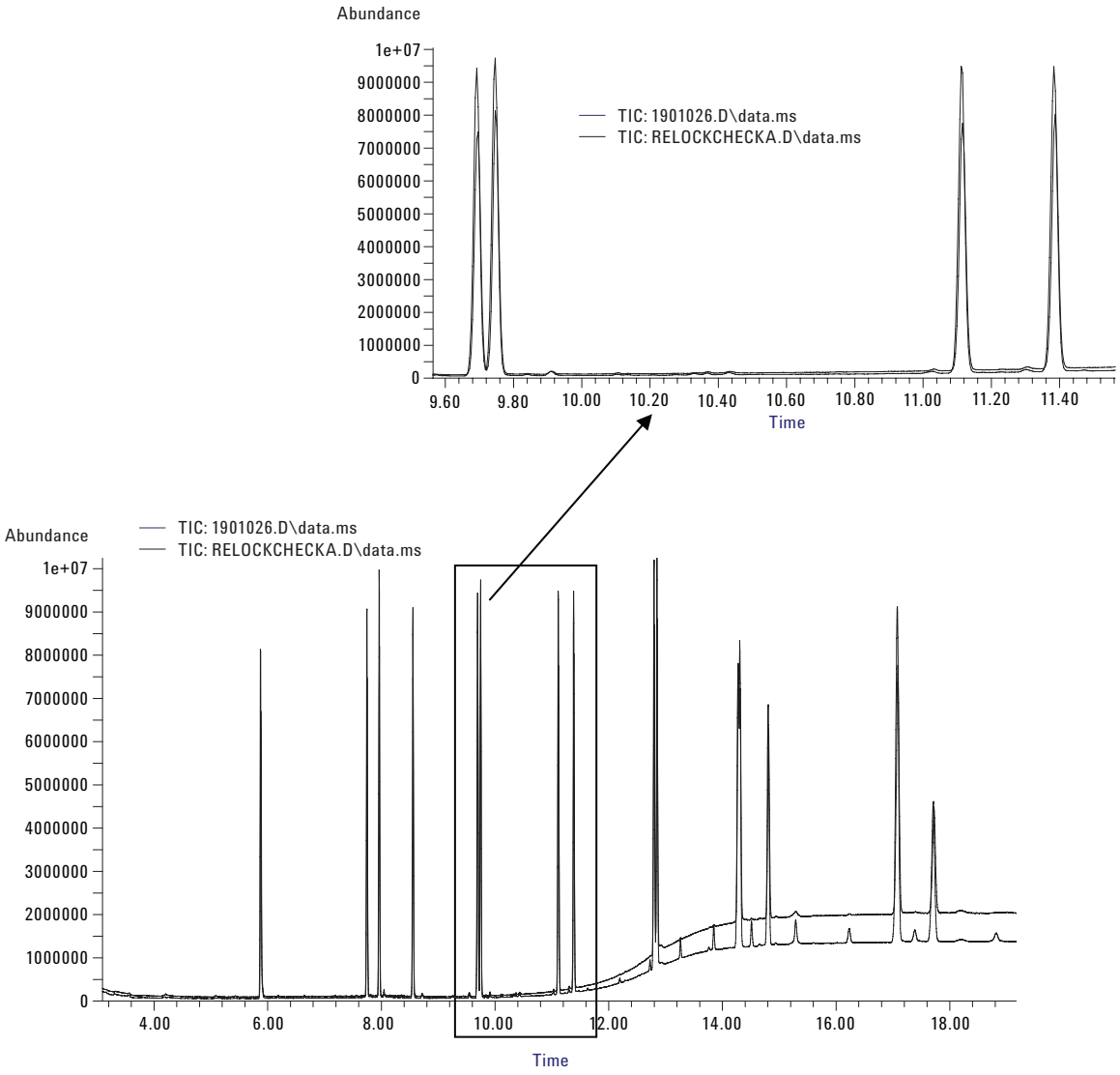



**Figure 4. Overlay of injections before and after column maintenance and relocking, including a zoomed area of the four peaks from phenanthrene to pyrene.**

## Conclusions

This application demonstrates the ability of the 7890 GC system to perform RTL at low pressures (sub 5 psi), such as those experienced when using a 320-µm column in a GC-MS system. The average retention time variation before and after column maintenance for a 16-PAH mixture is less than 0.5 sec, providing high confidence in peak assignments, even with critical separations.

## For More Information

For more information on our products and services, visit our Web site at www.agilent.com/chem.

**www.agilent.com/chem**

Agilent shall not be liable for errors contained herein or for incidental or consequential damages in connection with the furnishing, performance, or use of this material.

Information, descriptions, and specifications in this publication are subject to change without notice.

© Agilent Technologies, Inc. 2008

Printed in the USA
July 11, 2008
5989-8366EN

**Agilent Technologies**

# Case Study for Agilent Lab Monitor and Diagnostic Software

Application

**HPI, Environmental, Food Safety**

## Author

Chunxiao Wang
Agilent Technologies (Shanghai) Co. Ltd.
412 Ying Lun Road
Waigaoqiao Free Trade Zone
Shanghai 200131
China

## Abstract

**Agilent Lab Monitor & Diagnostic (LMD) software can monitor in real time all the Agilent GCs and LCs in your lab. It automatically tracks supply usage, monitors chromatographic quality, and notifies you of maintenance needs before a problem occurs by keeping track of injections, hours of operation, and other parameters that you specify. Two case studies are given demonstrating some of the features and benefits of this software.**

## Introduction

Agilent Lab Monitor & Diagnostic (LMD) software is an innovative tool to help you manage your lab to ensure performance, productivity, and reliability. LMD software can monitor in real time a single Agilent GC or LC or all the Agilent GCs and LCs in your lab. It automatically tracks supply usage, monitors chromatographic quality, and notifies you of maintenance needs before a problem occurs by keeping track of injections, hours of operation, and other parameters that you specify. LMD software "knows" when it's time to replace consumables or perform basic upkeep. It provides full diagnostic capabilities with an extended list of tests and calibration procedures and automates basic diagnostic routines that help verify proper instrument performance. The software is coupled with an extensive suite of user information Help functions that provide quick, easy access to maintenance information, such as manuals and videos, so that you can get the information you need right when you need it.

LMD provides the following features and benefits:

- Increases your lab's uptime by alerting you to problems before they happen
- Provides intuitive help with diagnostic capabilities and easy-to-follow repair procedures in case of a problem
- Enhances diagnostics and troubleshooting functionality with searchable, complete user information capability
- Keeps your systems in top condition and helps with routine troubleshooting
- Maximizes column and consumables utilization by optimizing replacement schedules
- Helps you meet regulatory requirements by keeping all maintenance, event, and run logs in a single, easily accessible location
- Provides a link to the optional, Web-enabled Remote Advisor to back up your internal service and support resources

The following two case studies demonstrate some of the features and benefits of the Agilent LMD software.

### Configuration for Case Studies

- One PC with LMD monitors two Agilent 7890A GCs
- Agilent LMD software (A.01.03, Advanced)
- Firmware (A.01.06)
- ChemStation (B.03.02)

The first case study demonstrates the feature/benefit of right advice/alarm by tracking resource counters before results go bad.

### Problem

Oxygen is an enemy to most capillary GC columns, especially polar columns. In this study, a polar column is used for analyzing trace oxygenates. The baseline is getting worse after 400 runs because of septum leakage, which may impact quantitative analysis for trace-level analysis (see Figure 1, upper chromatogram).

### Tracking Resource Counters and Giving Alarm Before Results Go Bad

Because LMD is configured to track the GC's inlet septum, it reminds the user to change the septum when the limit has been reached, before the GC baseline goes bad. After the septum is changed, the expected baseline is obtained. Figure 1 shows the baseline of the polar column after 400 runs (top) and after the septum has been changed (bottom).

LMD can track not only the inlet septum, but also other GC resources, like the inlet o-ring, oxygen traps, inlet gold seal, and so on. As Figure 2 shows, LMD gives real-time indicators and alerts of preventive maintenance needs.

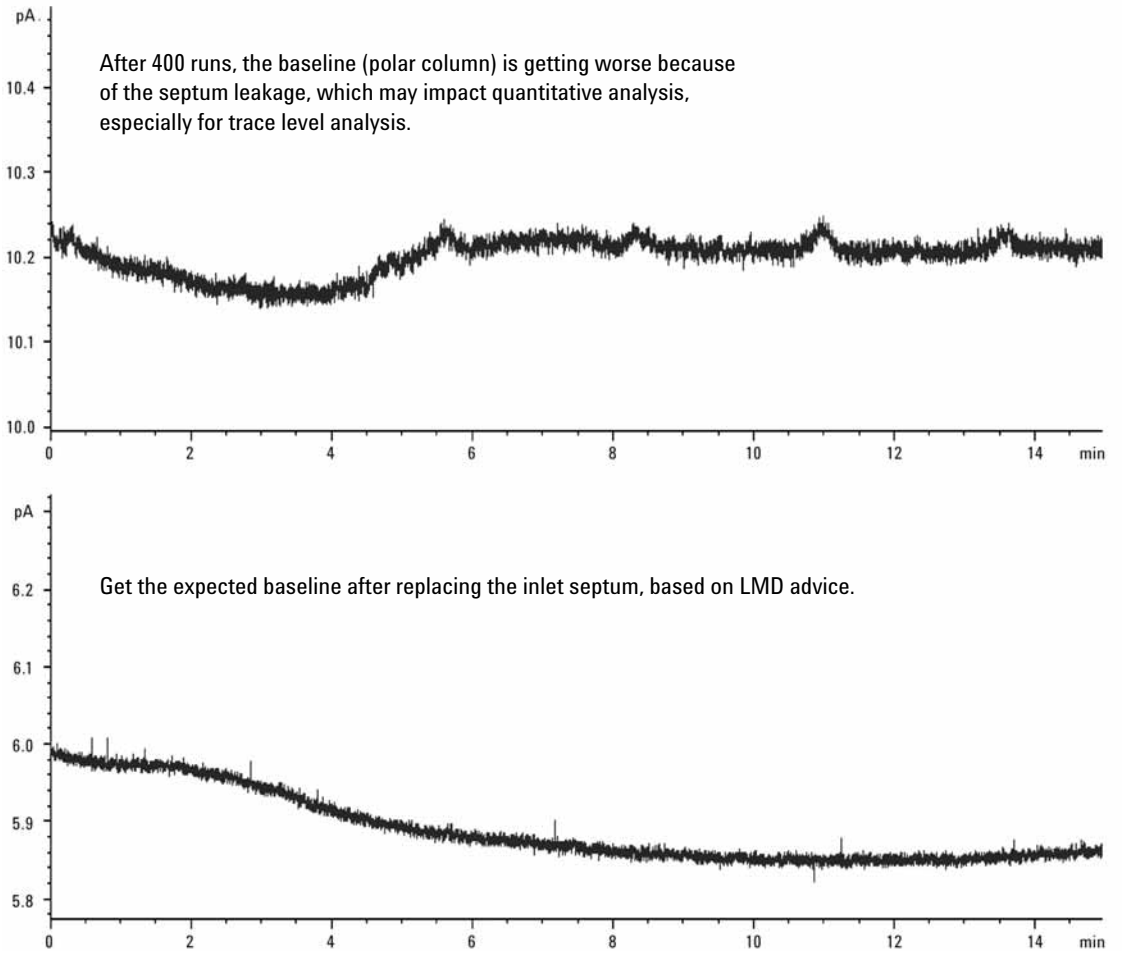



**Figure 1. Baseline chromatograms on polar column after 400 runs and after the inlet septum has been replaced.**

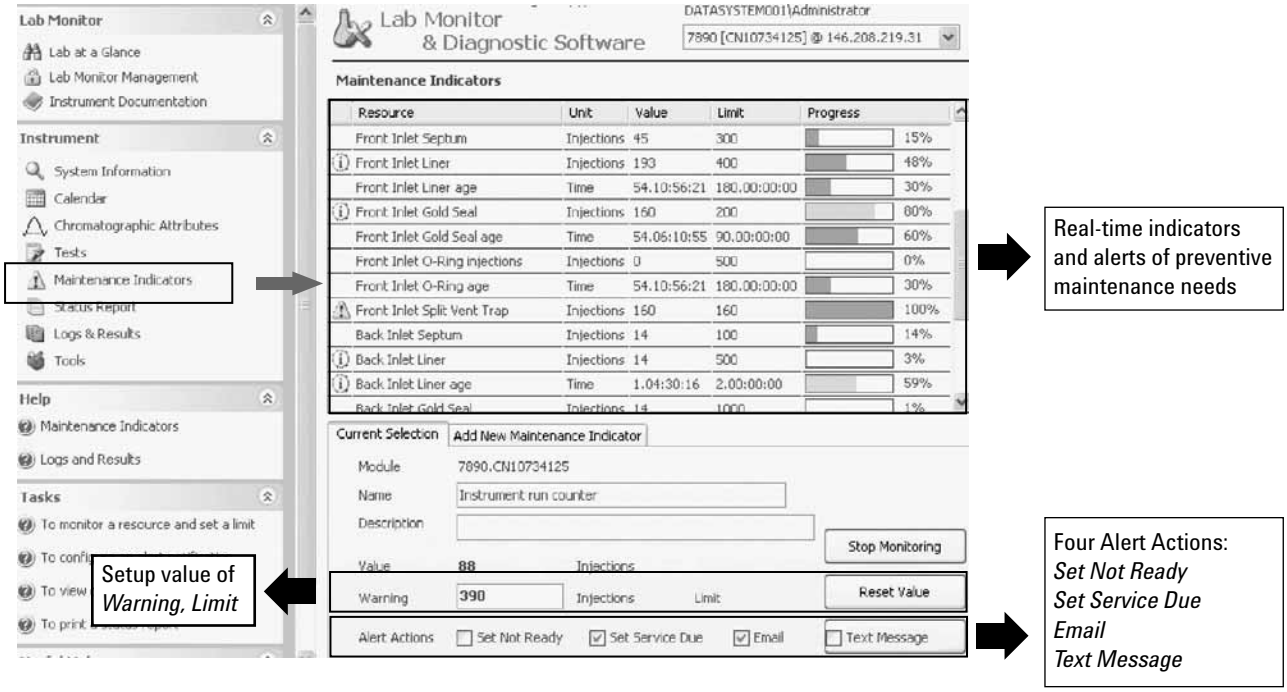

Lab Monitor
Lab at a Glance
Lab Monitor Management
Instrument Documentation
Instrument
System Information
Calendar
Chromatographic Attributes
Tests
Maintenance Indicators
Status Report
Logs & Results
Tools
Help
Maintenance Indicators
Logs and Results
Tasks
To monitor a resource and set a limit
Lab Monitor & Diagnostic Software
DATASYSTEM001\Administrator
7890 [CN10734125] @ 146.208.219.31
Maintenance Indicators
Resource | Unit | Value | Limit | Progress
Front Inlet Septum | Injections | 45 | 300 | 15%
Front Inlet Liner | Injections | 193 | 400 | 48%
Front Inlet Liner age | Time | 54.10:56:21 | 180.00:00:00 | 30%
Front Inlet Gold Seal | Injections | 160 | 200 | 80%
Front Inlet Gold Seal age | Time | 54.06:10:55 | 90.00:00:00 | 60%
Front Inlet O-Ring injections | Injections | 0 | 500 | 0%
Front Inlet O-Ring age | Time | 54.10:56:21 | 180.00:00:00 | 30%
Front Inlet Split Vent Trap | Injections | 160 | 160 | 100%
Back Inlet Septum | Injections | 14 | 100 | 14%
Back Inlet Liner | Injections | 14 | 500 | 3%
Back Inlet Liner age | Time | 1.04:30:16 | 2.00:00:00 | 59%
Current Selection
Add New Maintenance Indicator
Module 7890.CN10734125
Name Instrument run counter
Description
Stop Monitoring
Value 88 Injections
Warning 390 Injections Limit
Reset Value
Alert Actions
Set Not Ready
Set Service Due
Email
Text Message
Setup value of Warning, Limit
Real-time indicators and alerts of preventive maintenance needs
Four Alert Actions:
Set Not Ready
Set Service Due
Email
Text Message

**Figure 2. LMD real-time indicators and alerts of preventive maintenance needs.**

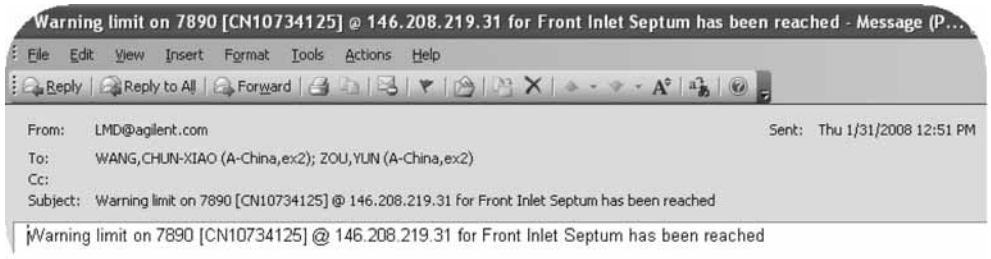

Warning limit on 7890 [CN10734125] @ 146.208.219.31 for Front Inlet Septum has been reached - Message (P...
File Edit View Insert Format Tools Actions Help
Reply Reply to All Forward
From: LMD@agilent.com
Sent: Thu 1/31/2008 12:51 PM
To: WANG,CHUN-XIAO (A-China,ex2); ZOU,YUN (A-China,ex2)
Cc:
Subject: Warning limit on 7890 [CN10734125] @ 146.208.219.31 for Front Inlet Septum has been reached
Warning limit on 7890 [CN10734125] @ 146.208.219.31 for Front Inlet Septum has been reached

**Figure 3. Alert email for when the front inlet septum limit has been reached.**

Four alert actions are available: Set Not Ready, Set Service Due, Email, and Text Message. For example, when the email alert action is selected, LMD will send an alert email to the users you specify. Figure 3 shows an example email alert informing a user that the front inlet septum's limit has been reached.

An email alert is configured in only three steps:

1. Manage the users (see Figure 4)
2. Manage the alerts (see Figure 5)
3. Configure the maintenance indicators

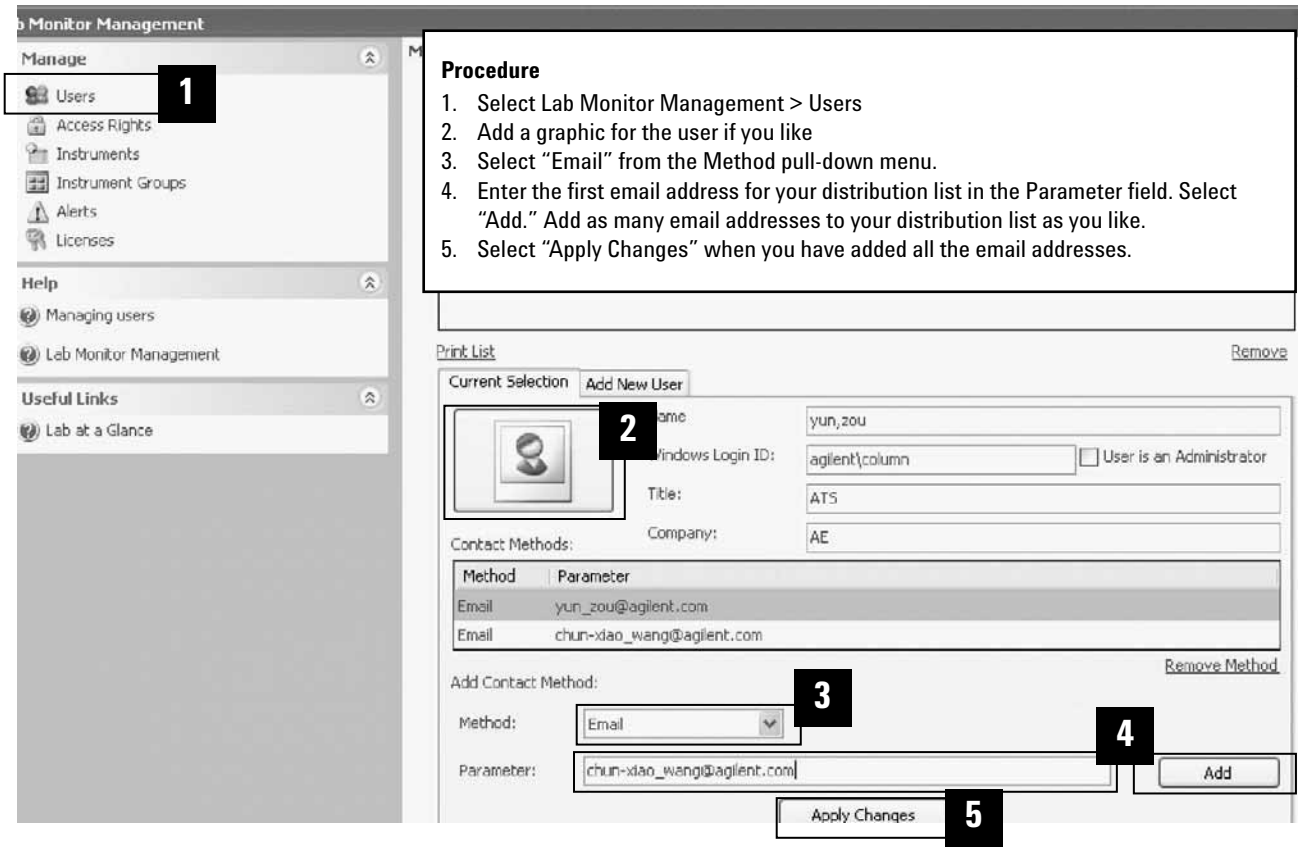



**Figure 4. Procedure for managing users.**

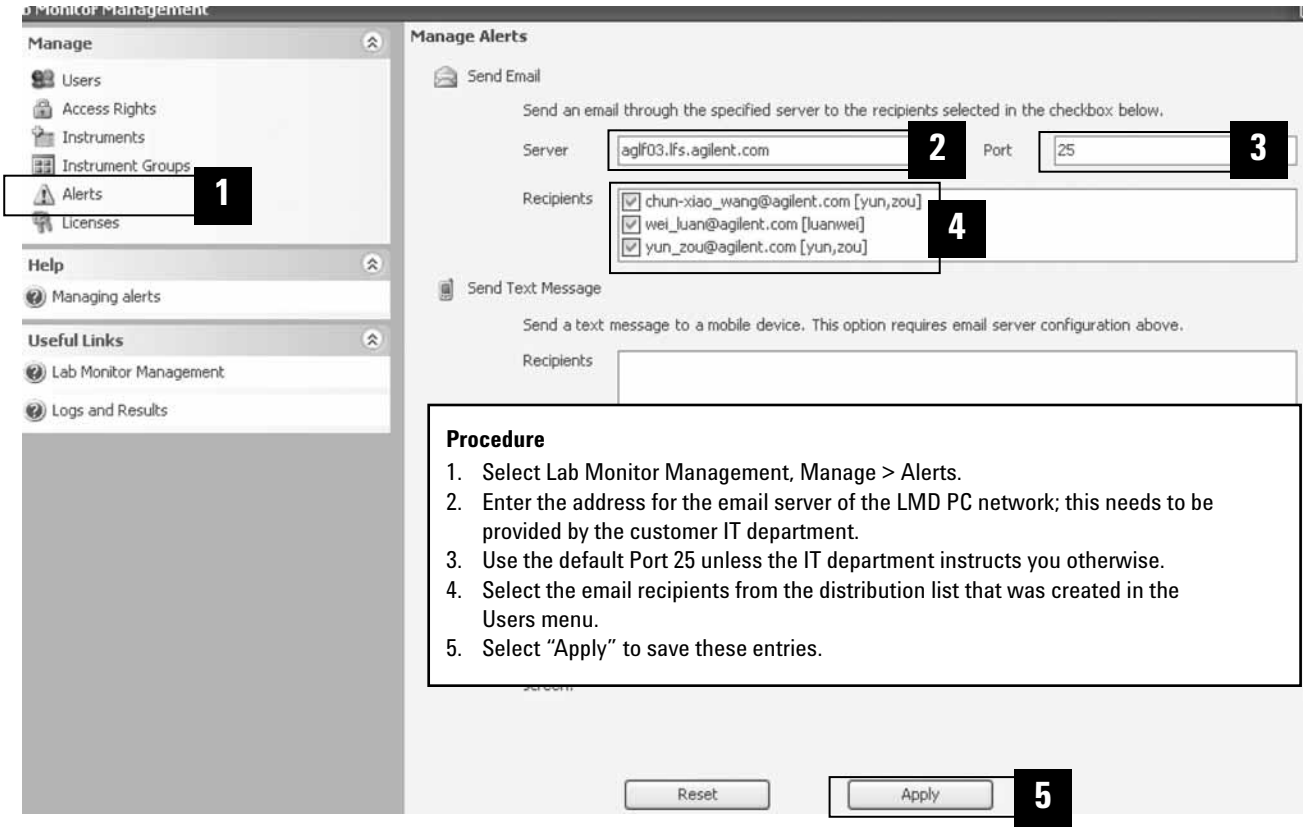



**Figure 5. Procedure for managing alerts.**

**Procedure**

1. Edit parameters and click the icon that allows for selection of diagnostic counters.
2. Select the counters that you want to use within LMD.

**Figure 6A. Procedure for configuring maintenance indicators. Set up the resource counter within ChemStation/Workstation.**

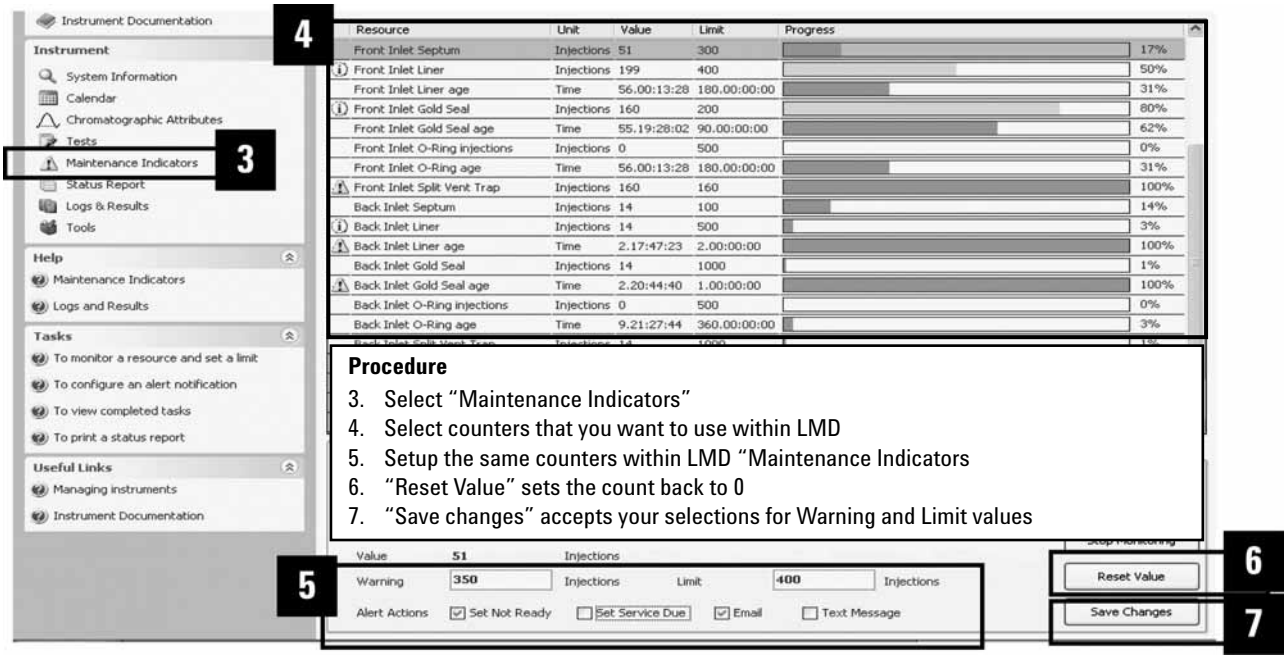

Instrument Documentation
Instrument
System Information
Calendar
Chromatographic Attributes
Tests
Maintenance Indicators
Status Report
Logs & Results
Tools
Help
Maintenance Indicators
Logs and Results
Tasks
To monitor a resource and set a limit
To configure an alert notification
To view completed tasks
To print a status report
Useful Links
Managing instruments
Instrument Documentation
3
4
Resource Unit Value Limit Progress
Front Inlet Septum Injections 51 300 17%
Front Inlet Liner Injections 199 400 50%
Front Inlet Liner age Time 56.00:13:28 180.00:00:00 31%
Front Inlet Gold Seal Injections 160 200 80%
Front Inlet Gold Seal age Time 55.19:28:02 90.00:00:00 62%
Front Inlet O-Ring injections Injections 0 500 0%
Front Inlet O-Ring age Time 56.00:13:28 180.00:00:00 31%
Front Inlet Split Vent Trap Injections 160 160 100%
Back Inlet Septum Injections 14 100 14%
Back Inlet Liner Injections 14 500 3%
Back Inlet Liner age Time 2.17:47:23 2.00:00:00 100%
Back Inlet Gold Seal Injections 14 1000 1%
Back Inlet Gold Seal age Time 2.20:44:40 1.00:00:00 100%
Back Inlet O-Ring injections Injections 0 500 0%
Back Inlet O-Ring age Time 9.21:27:44 360.00:00:00 3%
Procedure
3. Select "Maintenance Indicators"
4. Select counters that you want to use within LMD
5. Setup the same counters within LMD "Maintenance Indicators
6. "Reset Value" sets the count back to 0
7. "Save changes" accepts your selections for Warning and Limit values
5
Value 51 Injections
Warning 350 Injections Limit 400 Injections
Alert Actions Set Not Ready Set Service Due Email Text Message
6
Reset Value
Save Changes
7

**Figure 6B. Procedure for configuring maintenance indicators. Set up the same counter within LMD.**
In this example, a warning email will be sent at 350 injections on the front inlet liner. At 400 injections the GC will be set to "Not Ready," thus stopping the sequence, and an email will be sent to the distribution list that was set up in the previous procedure (see Figure 4).

For the 7890A GC, the resource counters must first be enabled from ChemStation before they will count within LMD (see Figure 6A). Next, the same counters are set up within LMD maintenance indicators (see Figure 6B).

The next case study demonstrates how LMD can provide intuitive help and easy-to-follow procedures in case there is a problem with diagnostic capabilities. It also illustrates how the complete, searchable user information enhances the diagnostic and troubleshooting functionality of LMD.

### Problem

The trace level peak was lost or its response reduced on the 7890A GC with FID and capillary column. In this scenario, the response of ethylbenzene is reduced and m-oxylene is lost, as shown in Figure 8.

### Designing Diagnostics and Tests to Solve Specific Problems

This problem may be caused by inlet leakage. The diagnostic tests of the LMD software simplify complex troubleshooting tests for the user by automatically performing specific troubleshooting-related tasks. For example, the inlet leak and inlet decay tests helps the user with quick diagnostics, and the FID checkout test helps verify proper performance with convenient guidance. The problem can be fixed efficiently with the intuitive help of LMD.

When an LMD test is running the GC Remote light comes on, indicating control of the GC. At this time, the ChemStation must be closed to run the LMD tests.

1. Run SS inlet 7890A leak check. Result: Failed. The procedures and result are shown in Figure 8.
2. Run SS inlet 7890A pressure decay test to confirm a leakage. Result: Failed. The above tests indicate that there is leakage in the inlet system. The procedures and result are shown in Figure 9.
3. Solve the inlet leak problem by tightening the column fittings, base gold seal fitting, split vent trap housing, septum nut, and the latch to the SSL inlet. Result: These actions don't work.
4. Replace the inlet septum and the liner O-ring. User document search capability makes it easy to find instructions on how to change the consumables, including the inlet septum and liner O-ring. Also, detailed consumable information, including part number, helps you select the right part (see Figure 10).
5. Run the SS inlet pressure decay and leak check tests. Result: Passed. See Figure 11.
6. Run the FID checkout test to confirm that the GC system is operating properly. Before the detector checkout test can run, you have to install the appropriate consumable parts; that is, an evaluation column, HP-5 30 m x 0.32 mm x 0.25 µm (p/n 19091J-413); an FID performance evaluation (checkout) sample (p/n 5188-5372) is also needed. The software will prompt you when you are required to perform a task or answer a question. When the FID detector checkout test is finished, restore the instrument to normal operating conditions. Result: Passed. See Figure 12.
7. Run trace-level aromatic sample. The expected result is obtained as shown in Figure 13 after diagnostics and troubleshooting.
8. The GC system is fixed.

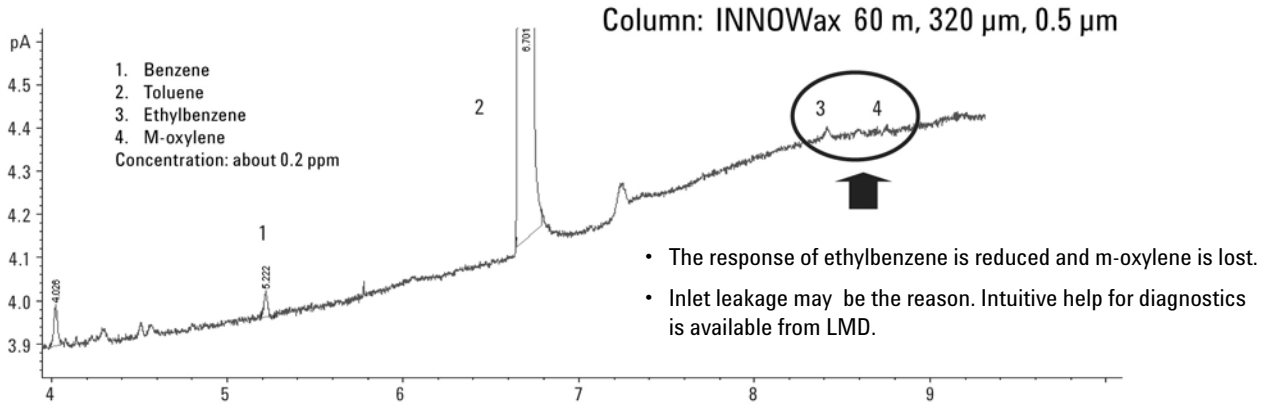



**Figure 7. Trace level peak lost or response reduced on 7890GC with FID and capillary column.**

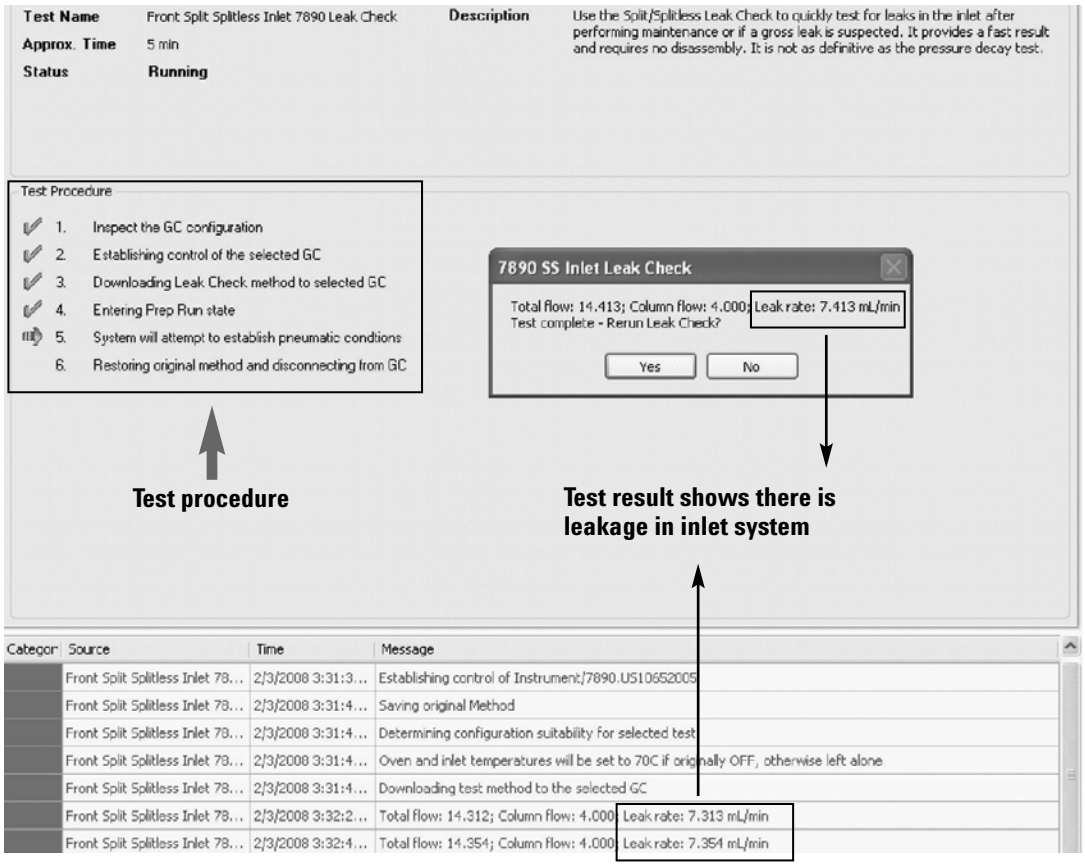

Test Name
Front Split Splitless Inlet 7890 Leak Check
Approx. Time
5 min
Status
Running
Description
Use the Split/Splitless Leak Check to quickly test for leaks in the inlet after performing maintenance or if a gross leak is suspected. It provides a fast result and requires no disassembly. It is not as definitive as the pressure decay test.
Test Procedure
1. Inspect the GC configuration
2. Establishing control of the selected GC
3. Downloading Leak Check method to selected GC
4. Entering Prep Run state
5. System will attempt to establish pneumatic conditions
6. Restoring original method and disconnecting from GC
7890 SS Inlet Leak Check
Total flow: 14.413; Column flow: 4.000; Leak rate: 7.413 mL/min
Test complete - Rerun Leak Check?
Yes
No
Test procedure
Test result shows there is leakage in inlet system
Categor
Source
Time
Message
Front Split Splitless Inlet 78...
2/3/2008 3:31:3...
Establishing control of Instrument/7890.US10652005
Front Split Splitless Inlet 78...
2/3/2008 3:31:4...
Saving original Method
Front Split Splitless Inlet 78...
2/3/2008 3:31:4...
Determining configuration suitability for selected test
Front Split Splitless Inlet 78...
2/3/2008 3:31:4...
Oven and inlet temperatures will be set to 70C if originally OFF, otherwise left alone
Front Split Splitless Inlet 78...
2/3/2008 3:31:4...
Downloading test method to the selected GC
Front Split Splitless Inlet 78...
2/3/2008 3:32:2...
Total flow: 14.312; Column flow: 4.000; Leak rate: 7.313 mL/min
Front Split Splitless Inlet 78...
2/3/2008 3:32:4...
Total flow: 14.354; Column flow: 4.000; Leak rate: 7.354 mL/min

**Figure 8. Procedure and result for SS inlet leak check test.**

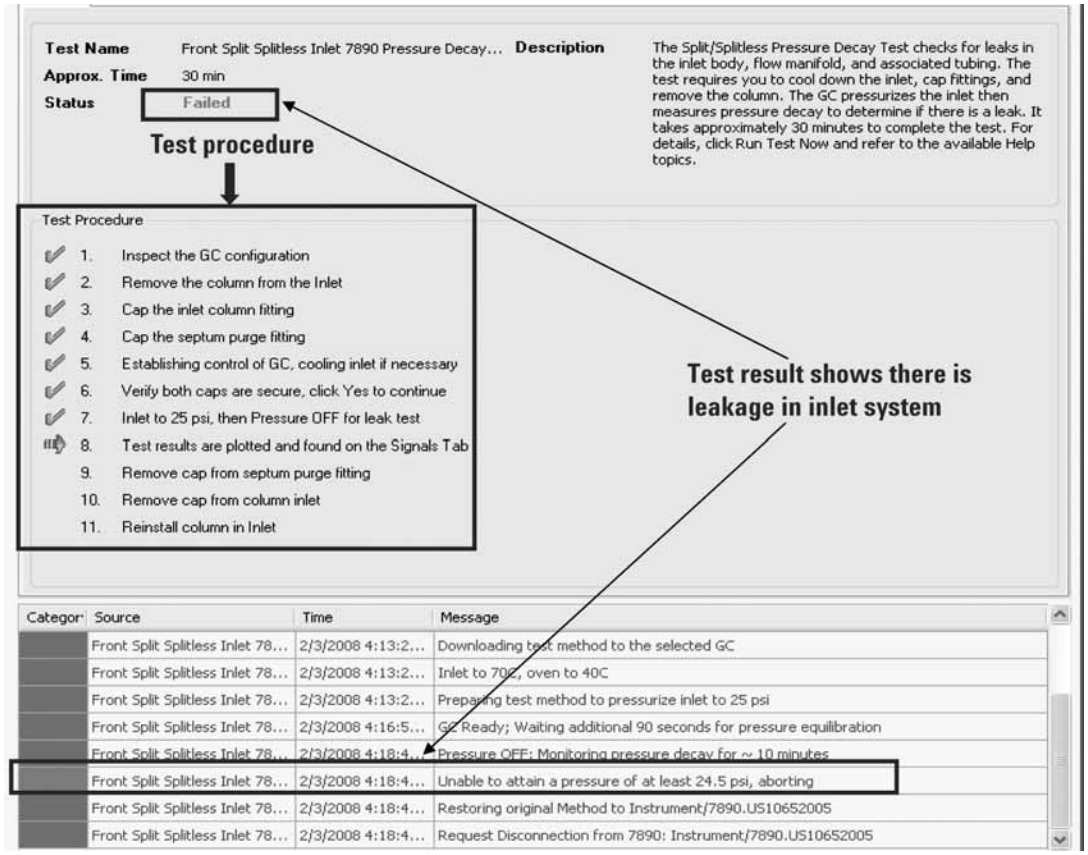
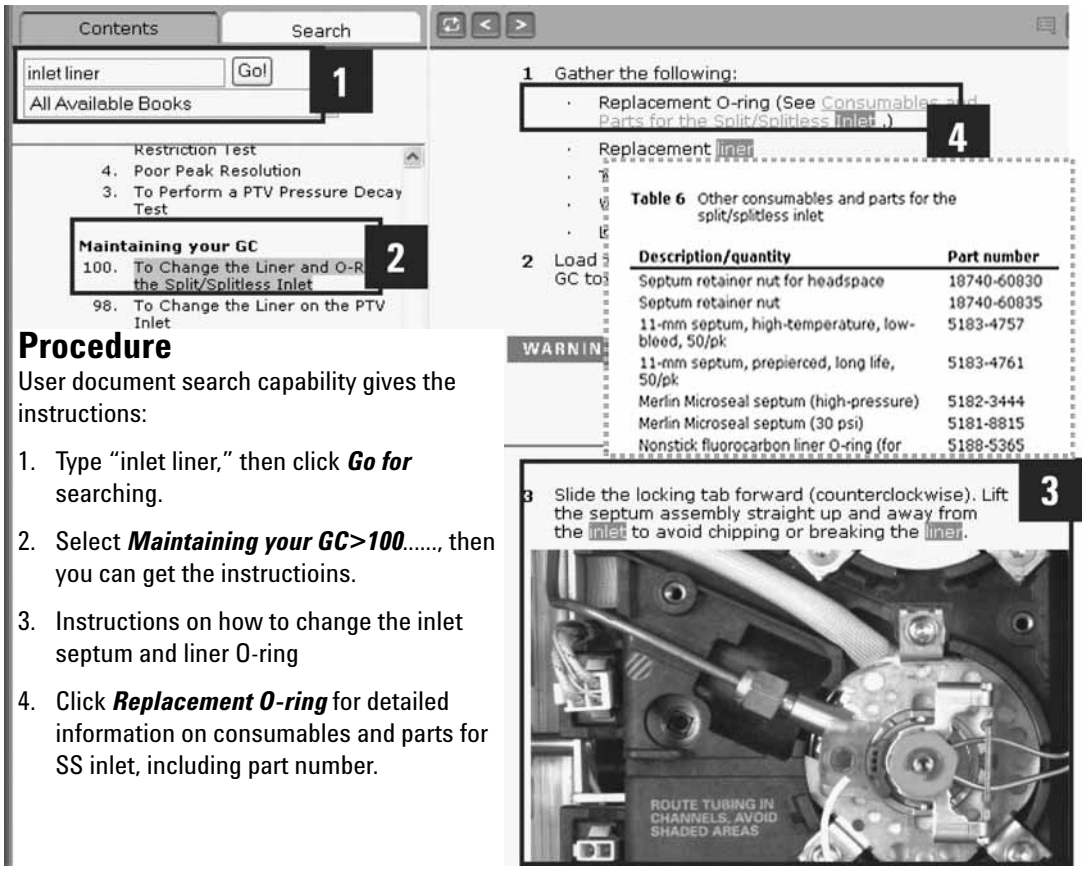

Test Name
Front Split Splitless Inlet 7890 Pressure Decay...
Approx. Time
30 min
Status
Failed
Description
The Split/Splitless Pressure Decay Test checks for leaks in the inlet body, flow manifold, and associated tubing. The test requires you to cool down the inlet, cap fittings, and remove the column. The GC pressurizes the inlet then measures pressure decay to determine if there is a leak. It takes approximately 30 minutes to complete the test. For details, click Run Test Now and refer to the available Help topics.
Test procedure
Test Procedure
1. Inspect the GC configuration
2. Remove the column from the Inlet
3. Cap the inlet column fitting
4. Cap the septum purge fitting
5. Establishing control of GC, cooling inlet if necessary
6. Verify both caps are secure, click Yes to continue
7. Inlet to 25 psi, then Pressure OFF for leak test
8. Test results are plotted and found on the Signals Tab
9. Remove cap from septum purge fitting
10. Remove cap from column inlet
11. Reinstall column in Inlet
Test result shows there is leakage in inlet system
Categor
Source
Time
Message
Front Split Splitless Inlet 78...
2/3/2008 4:13:2...
Downloading test method to the selected GC
Front Split Splitless Inlet 78...
2/3/2008 4:13:2...
Inlet to 70C, oven to 40C
Front Split Splitless Inlet 78...
2/3/2008 4:13:2...
Preparing test method to pressurize inlet to 25 psi
Front Split Splitless Inlet 78...
2/3/2008 4:16:5...
GC Ready; Waiting additional 90 seconds for pressure equilibration
Front Split Splitless Inlet 78...
2/3/2008 4:18:4...
Pressure OFF; Monitoring pressure decay for ~ 10 minutes
Front Split Splitless Inlet 78...
2/3/2008 4:18:4...
Unable to attain a pressure of at least 24.5 psi, aborting
Front Split Splitless Inlet 78...
2/3/2008 4:18:4...
Restoring original Method to Instrument/7890.US10652005
Front Split Splitless Inlet 78...
2/3/2008 4:18:4...
Request Disconnection from 7890: Instrument/7890.US10652005

**Figure 9. Procedure and result for SS inlet pressure decay test.**




## Procedure

User document search capability gives the instructions:

1. Type "inlet liner," then click ***Go for*** searching.
2. Select ***Maintaining your GC>100***....., then you can get the instructioins.
3. Instructions on how to change the inlet septum and liner O-ring
4. Click ***Replacement O-ring*** for detailed information on consumables and parts for SS inlet, including part number.

**Figure 10. Searchable, complete user information capability allows for enhanced diagnostics and troubleshooting functionality.**

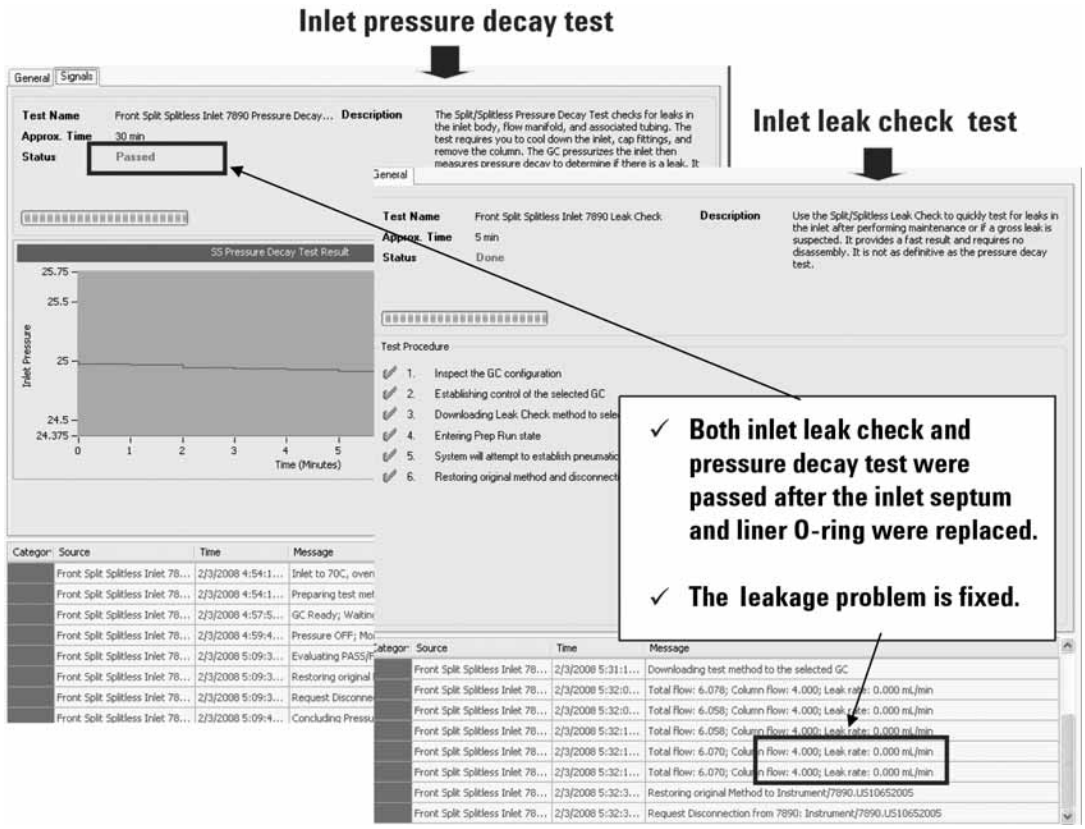

Inlet pressure decay test
Inlet leak check test
General
Signals
Test Name
Front Split Splitless Inlet 7890 Pressure Decay...
Description
The Split/Splitless Pressure Decay Test checks for leaks in the inlet body, flow manifold, and associated tubing. The test requires you to cool down the inlet, cap fittings, and remove the column. The GC pressurizes the inlet then measures pressure decay to determine if there is a leak. It
Approx. Time
30 min
Status
Passed
SS Pressure Decay Test Result
Inlet Pressure
Time (Minutes)
Test Name
Front Split Splitless Inlet 7890 Leak Check
Description
Use the Split/Splitless Leak Check to quickly test for leaks in the inlet after performing maintenance or if a gross leak is suspected. It provides a fast result and requires no disassembly. It is not as definitive as the pressure decay test.
Approx. Time
5 min
Status
Done
Test Procedure
1. Inspect the GC configuration
2. Establishing control of the selected GC
3. Downloading Leak Check method to sele
4. Entering Prep Run state
5. System will attempt to establish pneumatic
6. Restoring original method and disconnect
✓ Both inlet leak check and pressure decay test were passed after the inlet septum and liner O-ring were replaced.
✓ The leakage problem is fixed.
Source
Time
Message
Downloading test method to the selected GC
Restoring original Method to Instrument/7890.US10652005
Request Disconnection from 7890: Instrument/7890.US10652005

**Figure 11. The result passed both inlet leak check and pressure decay test after troubleshooting.**

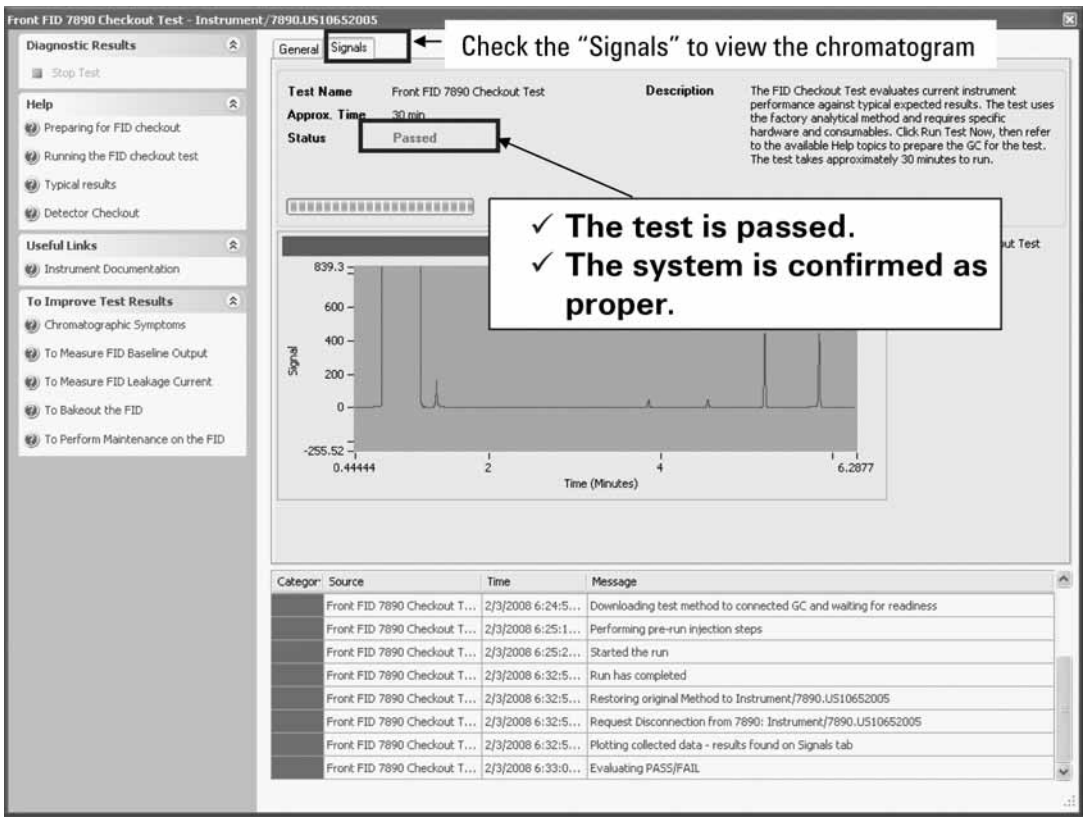

Front FID 7890 Checkout Test - Instrument/7890.US10652005
Diagnostic Results
Stop Test
Help
Preparing for FID checkout
Running the FID checkout test
Typical results
Detector Checkout
Useful Links
Instrument Documentation
To Improve Test Results
Chromatographic Symptoms
To Measure FID Baseline Output
To Measure FID Leakage Current
To Bakeout the FID
To Perform Maintenance on the FID
General
Signals
Check the "Signals" to view the chromatogram
Test Name
Front FID 7890 Checkout Test
Approx. Time
30 min
Status
Passed
Description
The FID Checkout Test evaluates current instrument performance against typical expected results. The test uses the factory analytical method and requires specific hardware and consumables. Click Run Test Now, then refer to the available Help topics to prepare the GC for the test. The test takes approximately 30 minutes to run.
✓ The test is passed.
✓ The system is confirmed as proper.
839.3
600
400
200
0
-255.52
Signal
0.44444
2
4
6.2877
Time (Minutes)
Categor
Source
Time
Message
Front FID 7890 Checkout T... 2/3/2008 6:24:5... Downloading test method to connected GC and waiting for readiness
Front FID 7890 Checkout T... 2/3/2008 6:25:1... Performing pre-run injection steps
Front FID 7890 Checkout T... 2/3/2008 6:25:2... Started the run
Front FID 7890 Checkout T... 2/3/2008 6:32:5... Run has completed
Front FID 7890 Checkout T... 2/3/2008 6:32:5... Restoring original Method to Instrument/7890.US10652005
Front FID 7890 Checkout T... 2/3/2008 6:32:5... Request Disconnection from 7890: Instrument/7890.US10652005
Front FID 7890 Checkout T... 2/3/2008 6:32:5... Plotting collected data - results found on Signals tab
Front FID 7890 Checkout T... 2/3/2008 6:33:0... Evaluating PASS/FAIL

**Figure 12. The result has passed FID checkout test.**

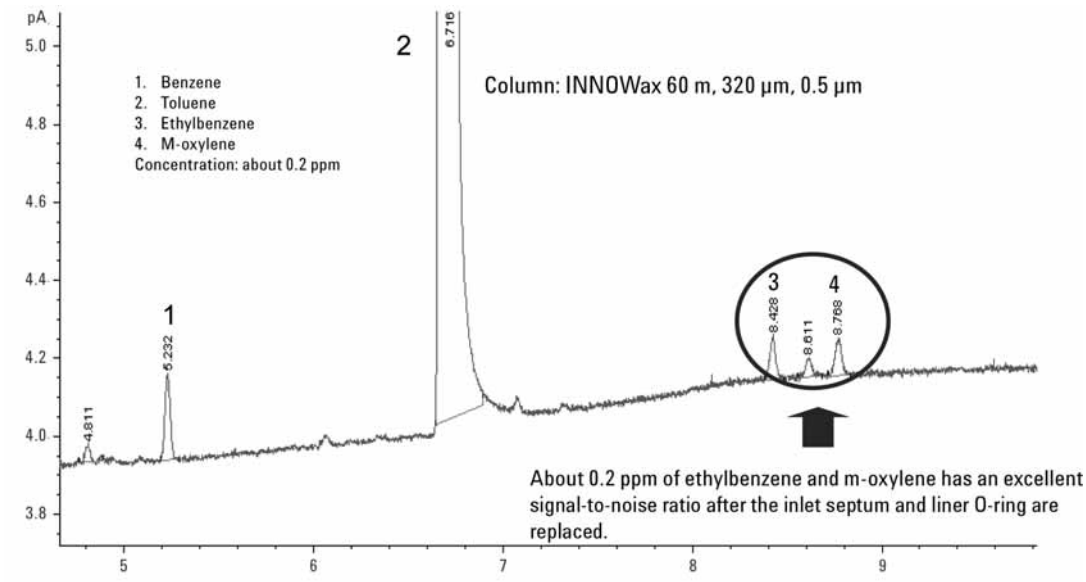



**Figure 13. Expected result for trace-level aromatic sample is obtained after troubleshooting with intuitive help from LMD.**

## Conclusions

Agilent Lab Monitor & Diagnostic (LMD) software is a new tool to help ensure the productivity, performance, and reliability of instruments in the lab. LMD monitors multiple instruments in the lab continuously in real time and detects maintenance needs and instrument problems before a problem occurs. These case studies demonstrated the benefits of Agilent LMD software for customers.

- **Right advice before results go bad**

  Real-time instrument monitoring tracks the number of runs or the life of GC resources, such as inlet septa, liners, and o-rings. Then, when the user-specified limit has been reached, LMD generates an Alert Action, for example, it sends an alert email to specified user(s).

- **Intuitive help in case of a problem**

  - Diagnostics and tests will help you solve problems and verify proper performance.

**www.agilent.com/chem**

  - Easy-to-use system tests, including an inlet leak test and inlet decay test, will help you do quick diagnostics.
  - A detector checkout test will provide con venient guidance to help you verify proper performance.

- **Extensive user document search capability**

  LMD offers easy-to-find and easy-to-use instructions on how to change the consumables, including the inlet septum and liner O-ring, and detailed consumable information, including part numbers, to help you select the right parts.

## For More Information

For more information on our products and services, visit our Web site at www.agilent.com/chem.

Agilent shall not be liable for errors contained herein or for incidental or consequential damages in connection with the furnishing, performance, or use of this material.

Information, descriptions, and specifications in this publication are subject to change without notice.

© Agilent Technologies, Inc. 2008

Printed in the USA
May 15, 2008
5989-8613EN

Agilent Technologies

# Capillary Flow Technology for GC/MS: A Simple Tee Configuration for Analysis at Trace Concentrations with Rapid Backflushing for Matrix Elimination

Application

**Environmental, Drug Testing, and Forensics**


## Author

Harry Prest
Agilent Technologies Inc.
5301 Stevens Creek Blvd.
Santa Clara, CA 95051
USA



## Abstract

**Capillary Flow Technology devices offer the potential to enhance GC/MSD operation and robustness. In operation, they can allow rapid service of the GC column and inlet, including liner and septum, without venting or subjecting the MSD to air. In terms of robustness, late eluting compoundscan be removed from the column by "backflushing," which forces components to retreat through the column into the injection port before they damage the MSD source or compromise the next analysis. This leads to higher analytical integrity as both the column phase and the MSD can be protected. This application describes a simple arrangement for Capillary Flow Technology devices that provides ventless maintenance features with highly accelerated backflushing and minimal losses in the MSD signal. This solution supports GC analysis in constant flow mode with pressure pulsed injections and is recommended for all MSD users (in both electron impact or chemical ionization modes), including those with diffusion pump systems.**


## Introduction

The introduction of Electronic Pressure Control (EPC) was a major advance for GC and especially GC/MS analysis. EPC allowed development of the constant flow mode of analysis, which generates chromatographic peaks of consistent width (time) and allows optimization of MS cycle times to meet either qualitative or quantitative requirements. Also, splitless injections gained pressure pulsing or ramped flow modes, which lowered the analytes' residence time in the hot injection port and confined the expansion of the injection solvent (avoiding overfilling of the liner). The power of this approach lead to continued evolution of EPC technology with the present state of the art represented in the new 7890A GC.

The recent addition of Capillary Flow Technology (CFT) devices has reinvigorated and recast Deans switching and other pressure control approaches to GC analysis. One such CFT device, the QuickSwap [1–3], provides two important capabilities to GC/MS:

1) The ability to service and/or replace the entire analytical column or the injection port liner and septum without venting the MSD (yet still retaining high vacuum integrity)

2) The ability to remove from the column late-eluting, highly retained components that elute after the target compounds of analytical interest by reversing the carrier flow direction through the column in what is called “backflushing.” With the oven temperature elevated and the flow reversed, these very high boiling interferences can be pushed off the column into the split vent and thereby prevent degradation of the column phase or the detector.

A schematic representation of the arrangement that makes this possible is shown in Figure 1.

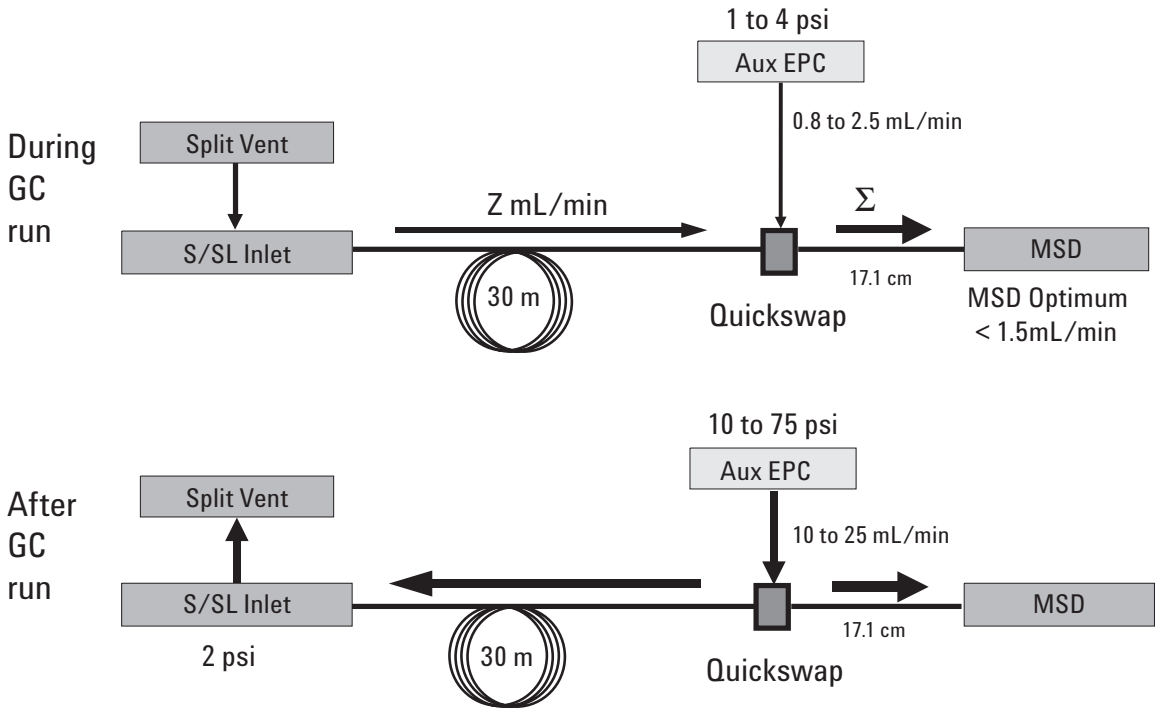



**Figure 1. Schematic of QuickSwap arrangement.**

Every new approach has a downside and for QuickSwap it is the additional makeup flow required to purge the QuickSwap device during analysis which dilutes the signal in the GC/MSD. This is not an issue for many users since the sensitivity of the MSD is usually more than adequate. However, analysis at trace concentrations has more stringent requirements and maintaining a signal closely comparable to that of a single continuous column is essential.

Another CFT configuration for GC/MSD applications designed specifically for trace GC/MS analysis where customers do not wish to surrender signal is possible using the QuickSwap or any of several other CFT devices. In this alternate configuration, the CFT device is located in the middle of the analytical column, essentially splitting the column in half. For example, a 15-m column preceeds and follows a CFT tee. Schematically this arrangement is illustrated in Figure 2. The auxiliary EPC device adds just enough pressure (flow) to match the flow (pressure) from the first column, so there is little flow addition and therefore less “dilution” and loss in the GC/MSD signal. Backflushing is similarly simple; the pressure or flow is dropped in the first column section while the second section column flow is increased.

Advantages of this pressure controlled tee (PCT) approach are similar to those of QuickSwap, such as:

- Service of injection port liner and septum without venting the MSD
- Column cutback or replacement of the “front” or first column without venting the MSD

But additional advantages of the PCT arrangement over QuickSwap are:

- Minimal or no signal loss (in EI- or CI-MS) is obtained because of the very small additional “makeup” gas flow.
- Constant flow mode and pressure pulsed injections are straightforward.
- This configuration is suitable for diffusion pumped systems and allows backflushing in diffusion pumped systems.
- Backflushing is more rapid and can be initiated earlier.

This application details some configurations and provides an example of backflushing.

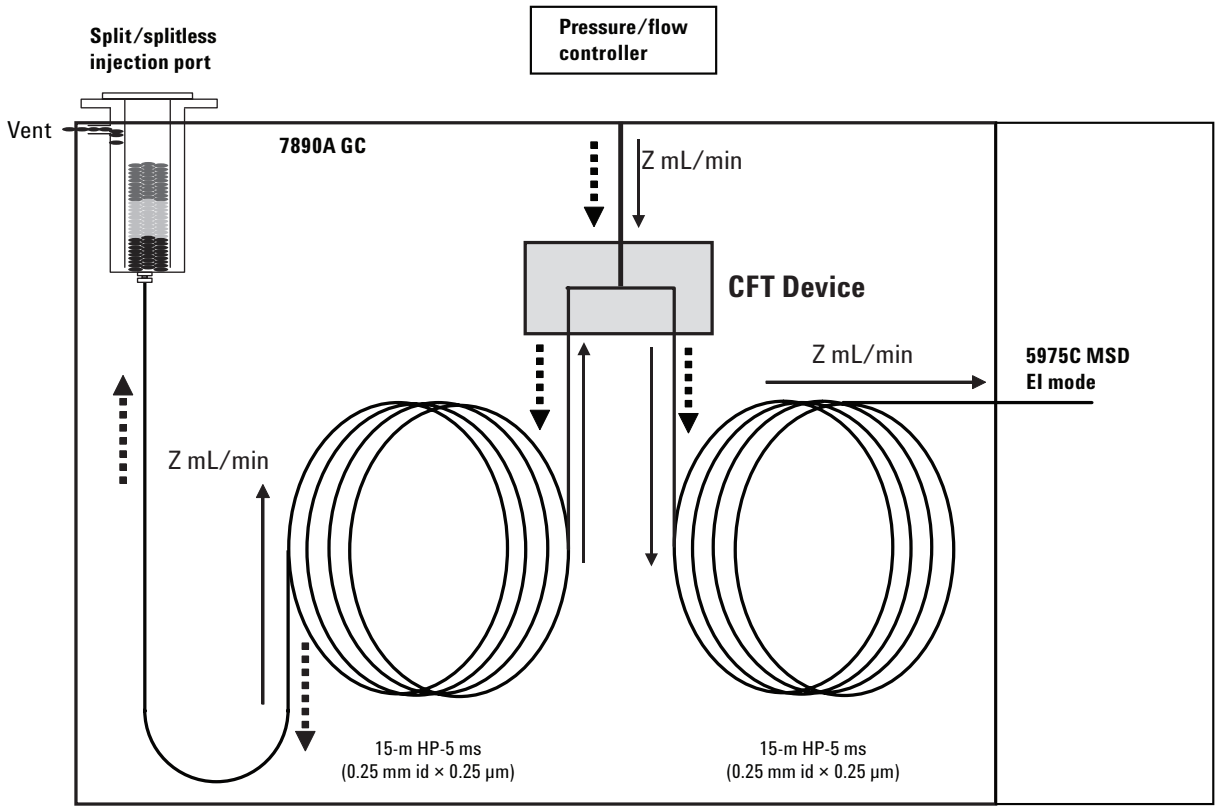



**Figure 2. Schematic of pressure controlled tee arrangement for the GC-MSD: solid lines indicate the forward flow during GC/MSD analysis and the dashed lines indicate backflushing flows.**

## Experimental

A number of devices can be used in this approach and those arrangements will be cited later, but for these experiments the instrument configuration was as follows:

- 7890A GC with split/splitless ports in front and back and a 7683B ALS
- 5975C MSD with performance turbomolecular pump
- 2 HP-5ms 15 m × 0.25 mm id × 0.25 µm film columns (19091S-431)
- CFT device: 2-way unpurged splitter (G3181-60500) with SilTite ferrules and nuts
- CFT GC mounting hardware: dual-wide mounting bracket (G2855-00140) or single-wide mounting bracket kit (G2855-00120)
- Deactivated 0.25 mm id column approximately 1 m long
- 2 CFT blanking plugs (G2855-60570 or as G2855-20550 with G2855-20593)

As an overview of the configuration, the 1-m column was connected to the back injection port and to the first position on the CFT splitter using the appropriate SilTite fittings. (This CFT device has three connection points and is really best thought of as a simple tee reminiscent of glass Y- or T-connectors and will be referred to as a "CFT device" or "CFT tee" from here forward).

One of the 15-m HP-5ms columns was connected at the uppermost position on the CFT tee and the other end through the transfer line into the MSD as usual. The other 15-m HP-5ms column was connected to the midpoint of the CFT device and the front injection port.

In detail, the arrangements were as follows. The CFT tee was attached to the forward position on the mounting hardware on the right side in the GC oven. The 1-m long section of guard column was wound on a spare column cage and hung on the column hanger in the back of the oven. (This could simply be added to one of the 15-m HP-5ms column

cages to avoid the extra cage.) Using a Vespel/graphite ferrule, one end was connected to the back injection port and the other end to the lowest connection of the CFT device with a SilTite ferrule and nut. The other two CFT tee connections were sealed with CFT blanking plugs and the back injection port was pressure tested as described in the 7890A *Advanced User Guide* (part number G3430-90015).

One of the 15-m columns was then hung on the cage carrying the 1-m column and installed with one end through the MSD transfer line. Since this column (column #2) can be expected to have a rather long life as it will be protected by the upstream column, a SilTite ferrule is recommended for the transfer-line seal. These ferrules do not develop leaks as the transfer-line temperature is cycled; however, the Vespel/graphite ferrules can shrink and develop leaks. (Note that if the surface of the transfer line is very worn it may fail to seal well, in which case the Restek *Agilent interface cleaner* [P/N 113450] can be used to resurface the sealing surface if very carefully employed). The other end of this GC column was connected to the uppermost connection on the CFT tee with the SilTite ferrule.

The "upstream" 15-m GC column (column #1) was hung on the other 15-m column cage and installed in the front split/splitless injection port with a Vespel/graphite ferrule, liner, and BTO septum, as usual. The other end was connected to the CFT tee middle post and, after temporarily removing the other connected columns, blanked off and pressure tested as above.

All connections were then re-established to the CFT tee with the 1-m column in the lower position; the front, first column (#1) connected in the middle position; and the rear, second or MSD column (#2) in the uppermost connection. Helium was supplied to both the front and back ports, and a helium leak detector was used to check for any leaks.

A picture of the arrangement is shown in Figure 3.

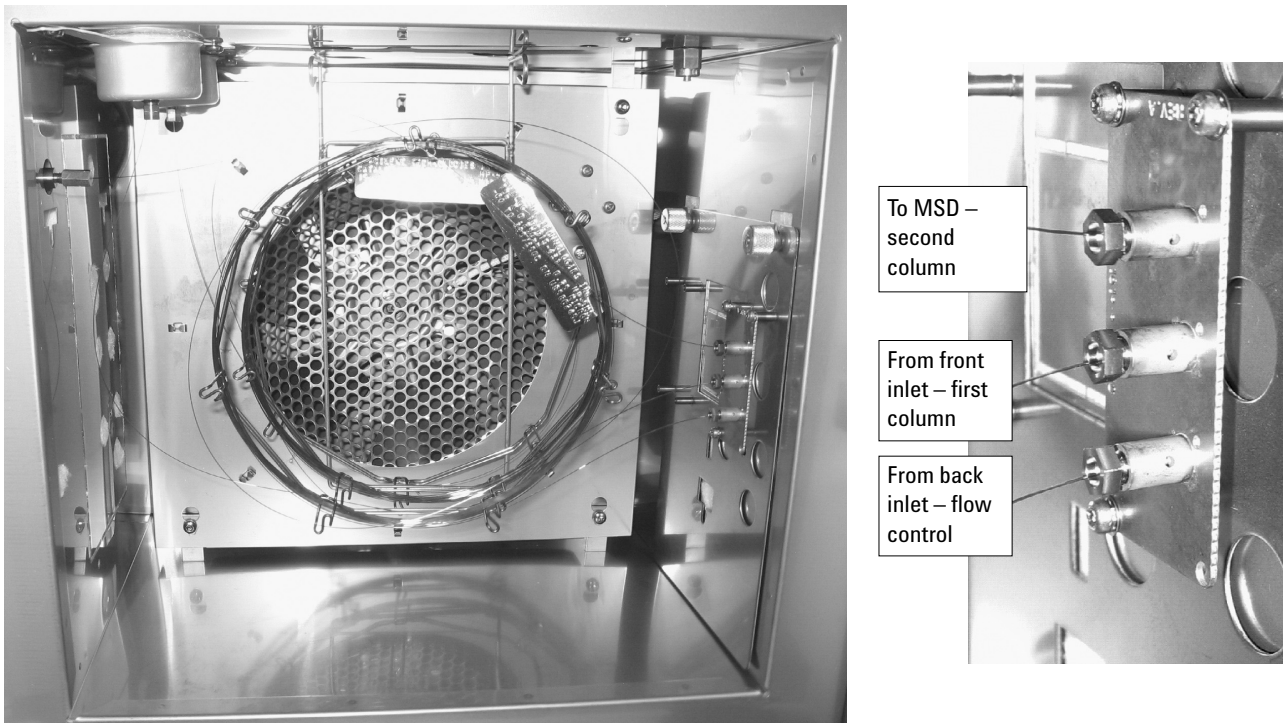



**Figure 3. Picture of the installed pressure controlled tee arrangement for the GC/MSD.**

### GC Configuration

The GC can be configured in several ways. However, for instructional purposes and those of these experiments, the GC was configured as follows:

| | |
|---|---|
| Column #1: | 30 m × 0.25 mm id × 0.25 µm column |
| Inlet: | Front injection port: pulsed splitless mode, split flow 15 mL/min |
| Outlet: | MSD (vacuum) |
| Mode: | Constant flow |
| Column #2: | 15 m × 0.25 mm id × 0.25 µm column |
| Inlet: | Back injection port: split mode, split flow 15-mL/min |
| Outlet: | MSD (vacuum) |
| Mode: | Constant flow |

The flows were set to 1.2-mL/min, all zones were left cold, and the MSD power was turned on. With the MSD and GC zones still "cold," the MSD background was checked to be sure *m/z* 28 was decreasing, indicating that the system was tight. Only after there was confidence that there was no leak were other zones brought up to temperature.

### Operating with Pressure Pulsed-Splitless Injection

Figures 4A and 4B show screen captures of the 7890A GC configuration for a standard pressure-pulsed splitless injection with constant flow mode operation; they show the front and back injection port parameters. Remember, the arrangement is set up such that the front port, into which the sample will be injected, is configured as if a 30-m column were installed into the MSD. Typical pressure-pulse conditions are set for these parameters: a 25 psi pulse for 0.5 minutes; split flow on at 0.75 minutes at 50-mL/min; with gas saver on at 2 minutes at 15-mL/min. The general rules apply for pressure-pulsed splitless injections: given a particular liner, inlet temperature, injection volume, and solvent, the expansion of the solvent is confined to a fraction of the interior volume (< 0.75) of the liner by the pressure applied.

Figure 4B shows that the back injection port is in split mode, at 120 °C (to remove water background), with split flow and gas saver set at 15-mL/min flow.

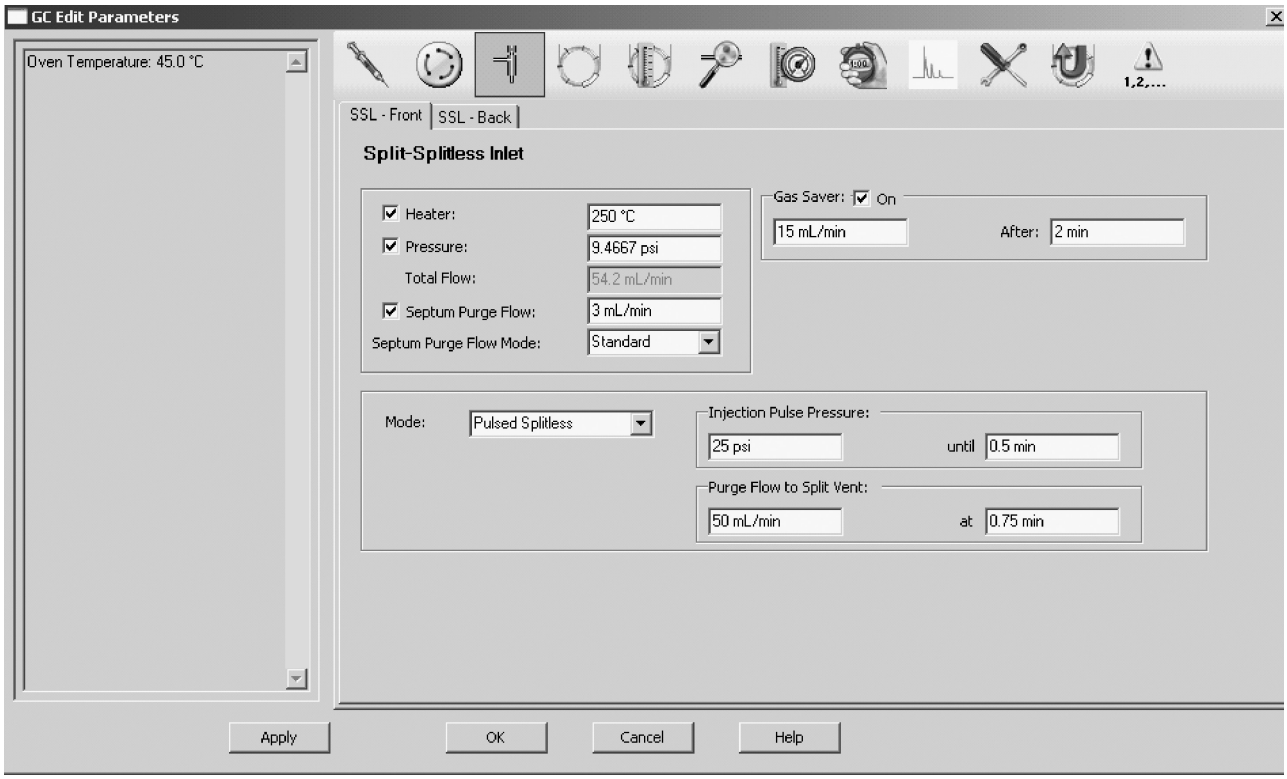



**Figure 4A (upper panel). Typical pressure-pulsed splitless injection parameters for constant flow: front injection port.**

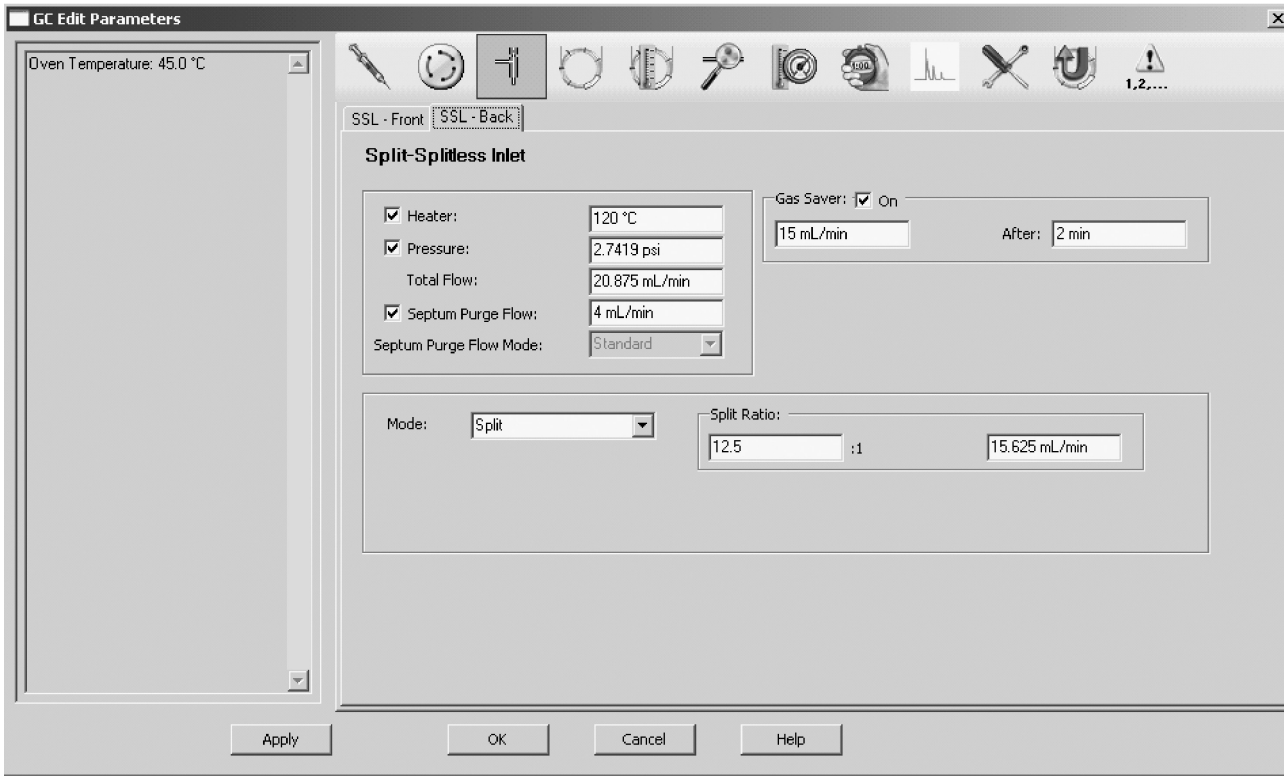



**Figure 4B (lower panel).** **Typical pressure-pulsed splitless injection parameters for constant flow: back injection port (not used for injection but for column control).**

Figures 5A and 5B show the constant flow mode settings for the two columns. The front column flow is the typical 1.20 mL/min, but the back column flow is slightly higher at 1.25 mL/min to prevent any backflow. Essentially the additional flow is equivalent to an extra meter of column length.

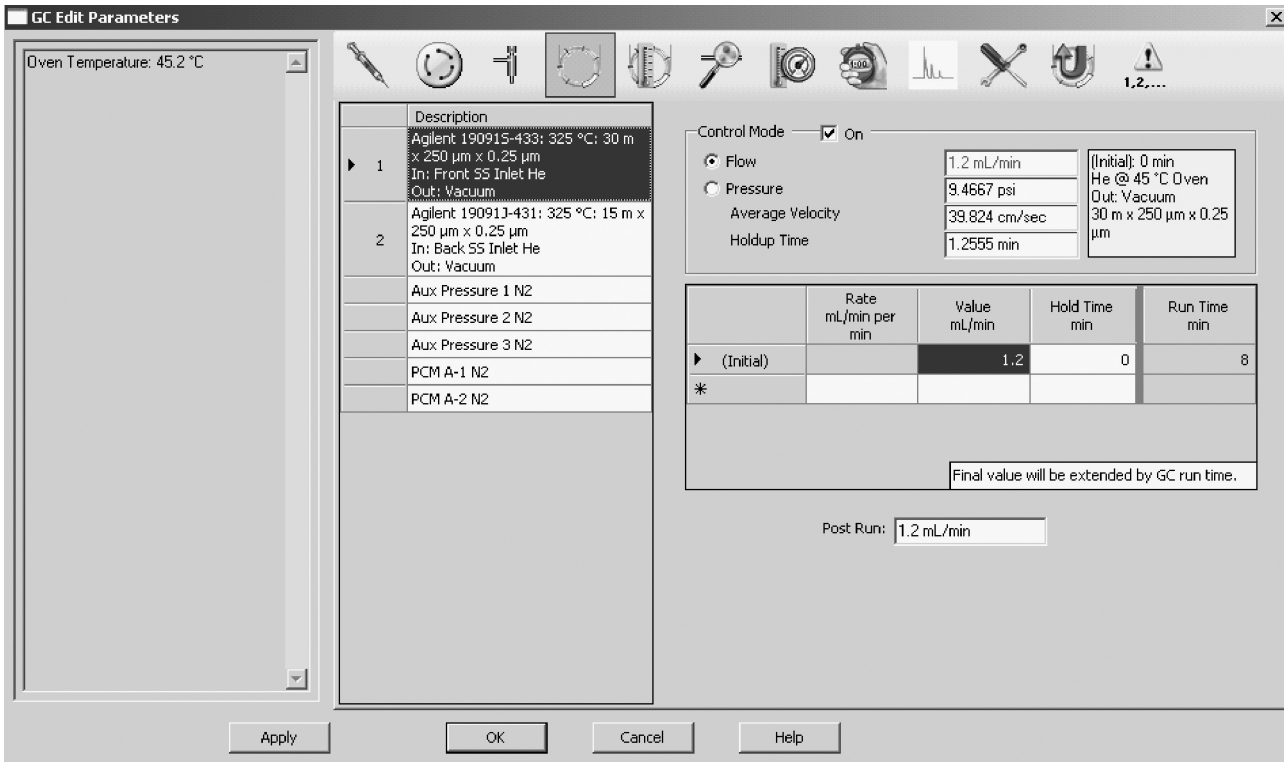



**Figure 5A (upper panel).** **Typical pressure-pulsed splitless injection parameters for constant flow: First column section (configured as a 30-m column).**

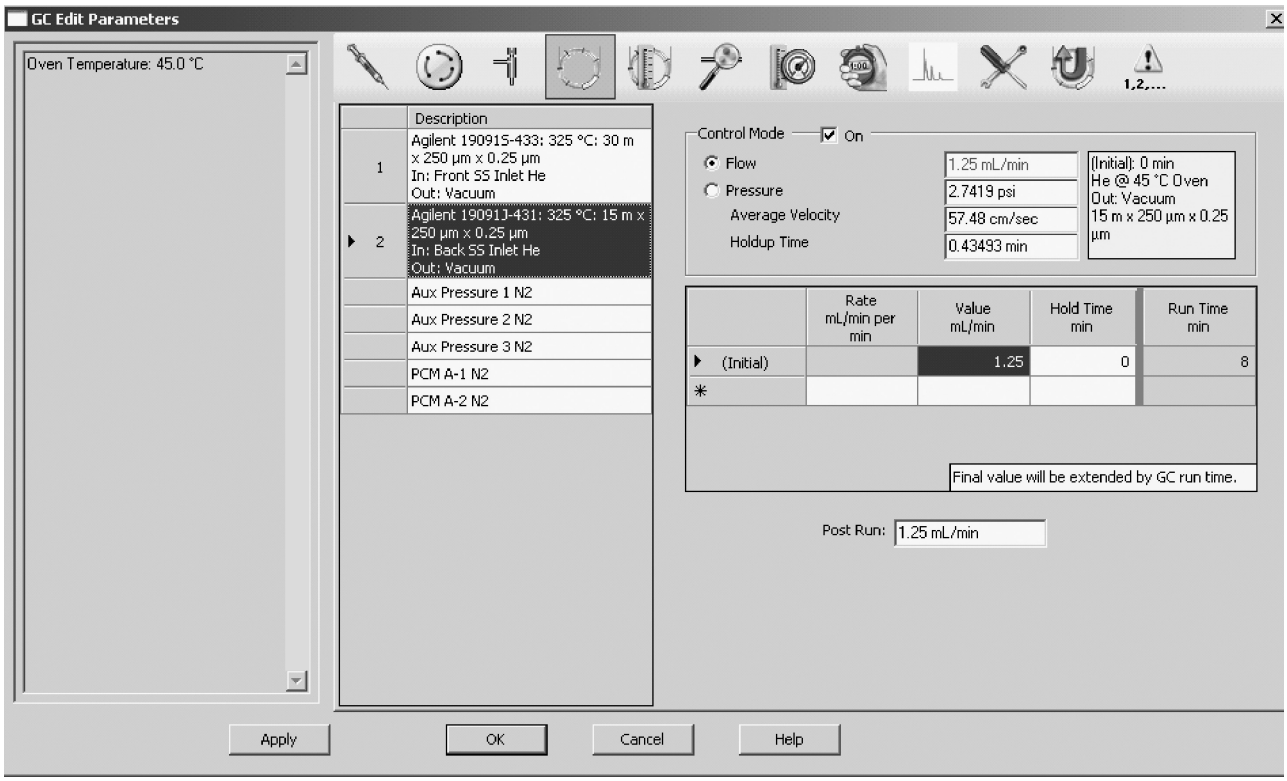



**Figure 5B (lower panel). Typical pressure-pulsed splitless injection parameters for constant flow: Second column section (configured as a 15-m column).**

## Results and Discussion

Figure 6 shows the results for pressure-pulsed splitless injections of octafluoronaphthalene (OFN) at 1-pg/µL acquired in selected ion monitoring (SIM) with the two 15-m column and CFT tee configuration and the standard 30-m continuous column configuration. Both peak height and area remain the same, indicating that there is no loss in signal. This is as expected since no signal dilution is taking place. There is a slight degradation in S/N for the CFT tee results as the background noise is raised by about 35% due to the additional flow controller. The important point is that the signal is preserved at trace levels.

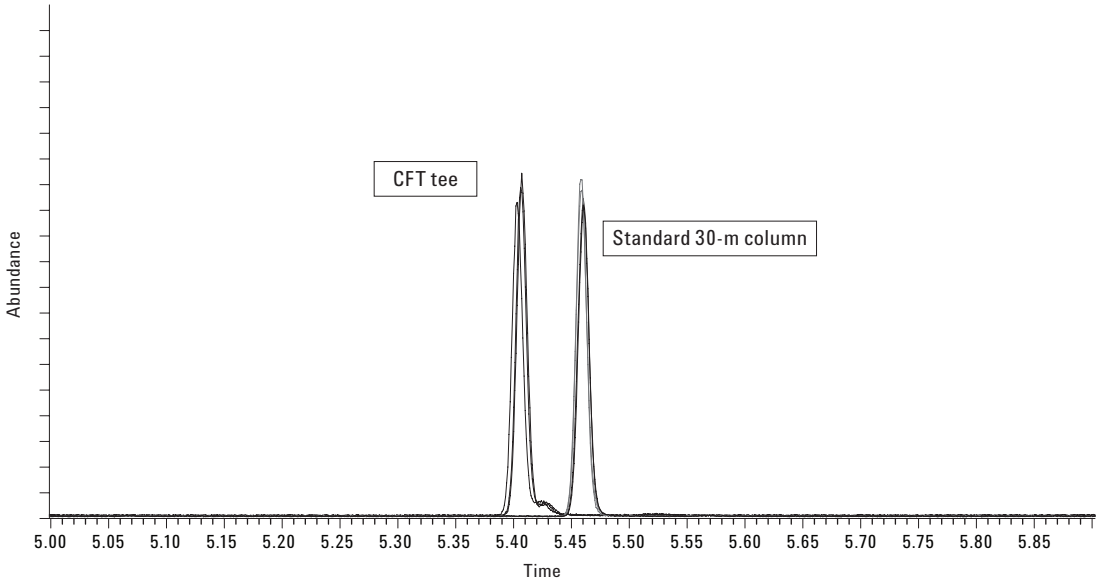



**Figure 6. Reconstructed total ion chromatogram (RTIC) of three replicate SIM acquisitions of octafluoronaphthalene using pulsed splitless injection with CFT tee (left profiles) and with a standard 30-m continuous column configuration (right profiles).**

### Chromatographic Character

Beyond preserving signal, the CFT device should exhibit reasonable chromatographic performance. One indication of chromatographic integrity is the peak shape profiles of the fatty acid methyl esters (FAMEs). The result for GC/MS analysis of a FAMEs standard acquired using a metabolomics method is shown in Figure 7 and suggests very little degradation of chromatography using this PCT. This can be expected as the path is deactivated and the path length in the channels in the PCT relative to the linear velocity suggests a relatively rapid transit through the device.

Another common chromatographic test used in organochlorine pesticide analysis (as in USEPA method 8081) examines degradation of 4,4'-DDT and Endrin. This degradation test was developed to indicate the degree of activity of the injection port by examining the amounts of DDD and DDE products of DDT and the ketone and aldehyde products of Endrin. The situation is complicated here as the degradation products can be generated in both the injection port and the CFT tee. How-

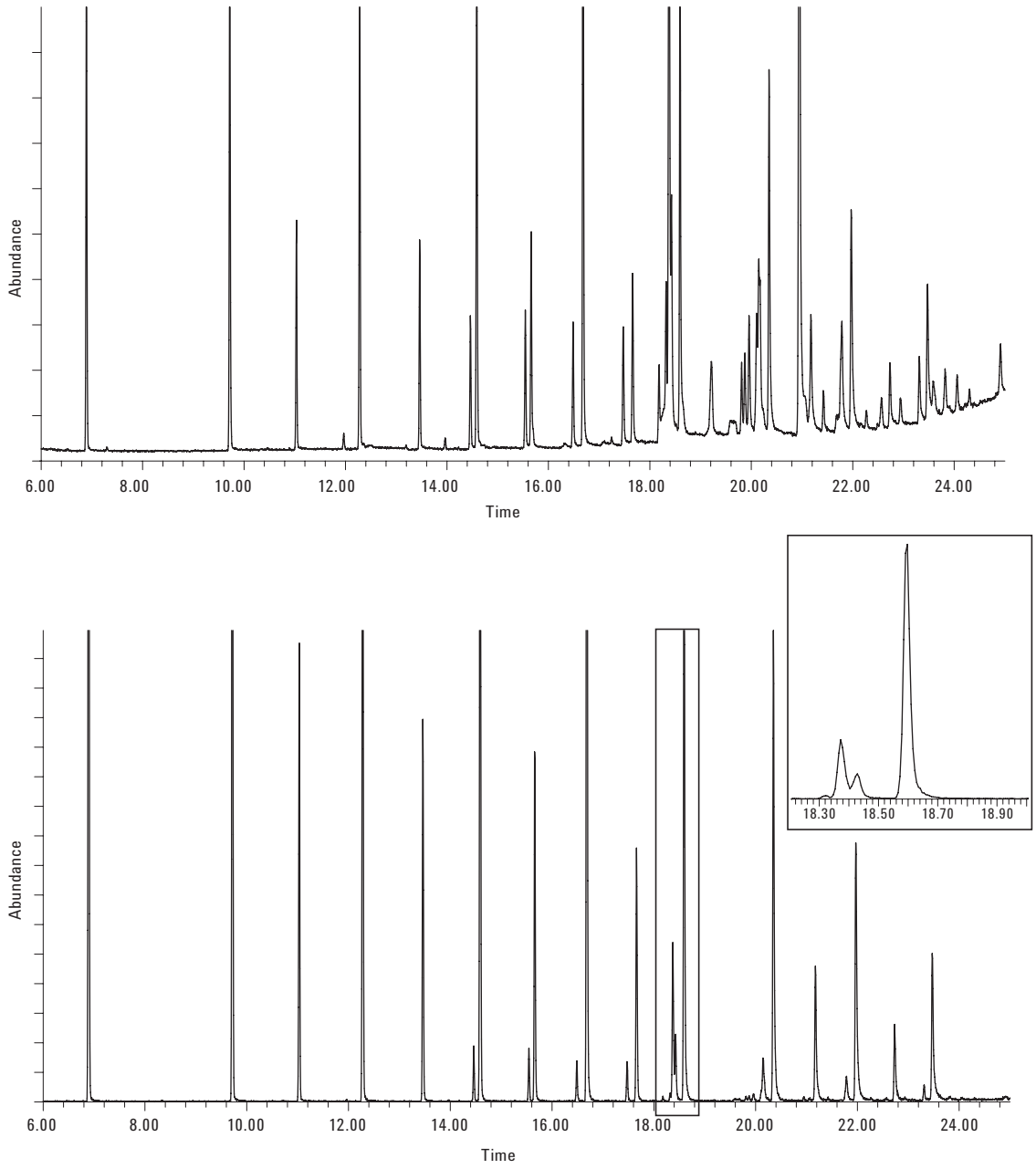



**Figure 7. Reconstructed total ion chromatogram (RTIC) of a multicomponent FAMEs standard using pulsed splitless injection with CFT tee (upper) and the reconstructed extracted ion chromatogram (REIC) for *m/z* 74. The enlarged panel is for octadecanoic methyl ester.**

ever, because those products formed in the injection port and those formed at the CFT device will have different retention times due to differing lengths of column, the degradation contributions from the two origins should be discernable. By analyzing these known breakdown products in the PCT and then injecting the DDT and Endrin agents themselves, an estimate of the activity contributed by the CFT device can be calculated. The upper panel of Figure 8 presents the reconstructed total ion current (RTIC) for the selected ion monitoring (SIM) signals of the four breakdown products. These were acquired in SIM-scan mode with a single SIM group composed of one or two major ions for each compound so there was no time selection for the compounds' appearance. On the basis of summed areas, the total breakdown for Endrin is less than 13% with the CFT device contributing less than 10% of the total breakdown area or less than 1.2% to the area total. The DDT breakdown is less than 4% for the system; however, the CFT device contributes about 46% of the total observed breakdown and is about double the breakdown generated by the port. It is possible some DDD breakdown is "hidden" under the DDT peak. On the basis of the DDT to DDE contribution from the CFT tee, however, it is likely to increase the breakdown perhaps less than about another 2%. A better study would use on-column injection

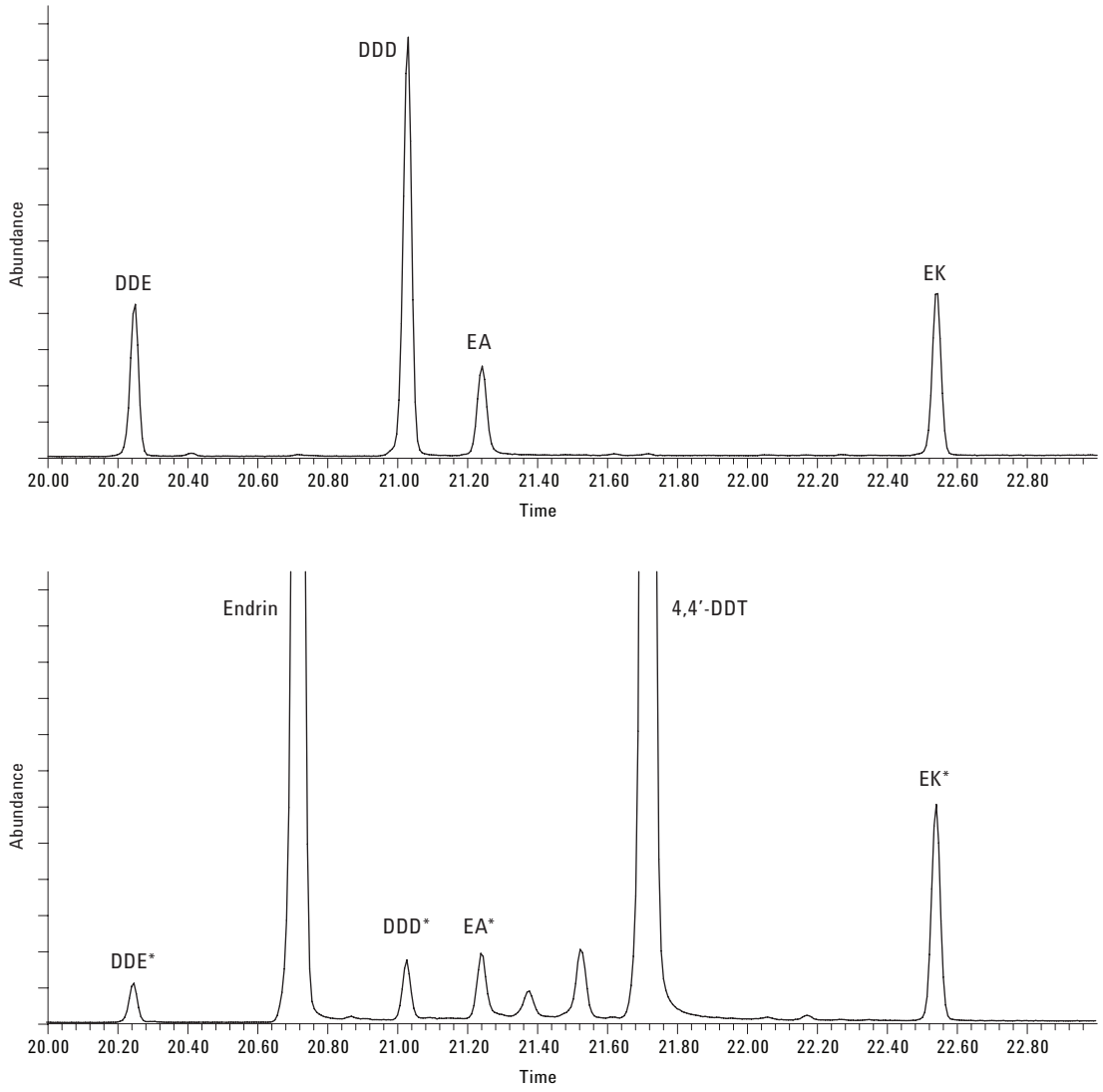



**Figure 8. CFT tee activity. A: the REIC of a GC-MS SIM acquisition using pulsed splitless injection with the PCT configuration of the expected degradation products of DDT and Endrin at 0.2 ng on column : 4,4'-DDE (DDE), 4,4'-DDD (DDD), Endrin aldehyde (EA), and ketone (EK). B: REIC for an injection of 2.0 ng of 4,4'-DDT and Endrin identifying degradation products. Those with an asterix (*) are attributed to the injection port and due to the CFT device activity such as; from Endrin (5 as ketone) and from 4,4'-DDT (6 as DDE). Note 7 is tenatively identified as DDMU, source unknown.**

of all components, but the verdict is likely the same: the CFT device has some activity but is comparable to that of other elements (for example, in the inlet and liner). It is worth noting that this CFT device has a very long path compared to others (see the *Alternative Configurations* section) and that air intrusion in any part of the system will be a major issue in considering activity problems.

### Adding Backflush

Figures 9A, 9B, and 9C show the GC parameters for adding backflush. They are quite simple. The oven temperature can remain the same as the temperature at the end of the oven program or can be raised to the isothermal or programmed temperature limits in Post Run for backflushing. Raising the column temperature during Post Run helps condition the column and removes some column bleed but is not necessary. The front column (column #1) flow is dropped to 0.3 mL/min and the back column (column #2) flow is raised to 4 mL/min.

To quickly estimate the duration of the Post-Run time parameter, notice that the back column (column #2) in Figure 9C cites the column Holdup Time at a given flow. At the 1.25-mL/min shown, the Holdup Time is roughly 0.4 minutes. When the column #2 flow is raised to 4 mL/min, the Holdup Time for back flow through column #1 will be less than this (actually around 0.26 min). But estimating that every 0.4 minute the front 15-m column section would be flushed at least once is very conservative and an adequate approximation. Five to 10 column volumes will flush this front 15-m section in less than 2 to 4 minutes, which is relatively rapid. Choose a time in this range (for example, 3 minutes) and test the effectiveness of the backflush method by injecting a sample and follow this with a solvent blank injected under the non-backflush GC/MSD method. There should be no sign of carryover. Extend this Post-Run time if there is carryover or further raise the Post-Run temperature or both. This is a very conservative approach.

### Column or Inlet Servicing and Maintenance

To change the liner, septum, cutback the column, or replace the front 15-m column, simply cool the inlet(s) and increase the flow on the back column (column #2) to 4 mL/min and set the front injection port pressure to OFF. It is worth saving this method (such as SERVICE-Front.M). When the head of the column is removed from the injection port, one can confirm that the carrier is flowing back up the column by immersing the tip in liquid.

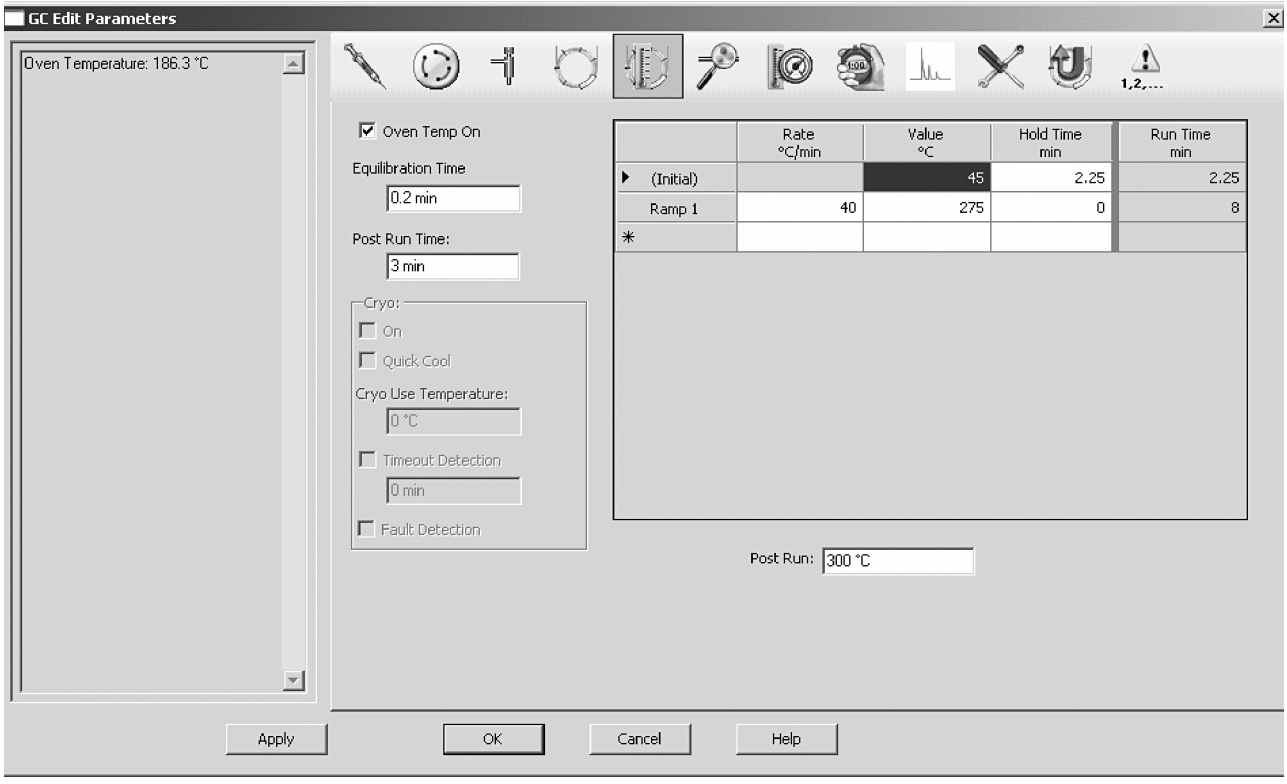



**Figure 9A (upper panel). Adding backflushing in Post Run: oven parameters.**

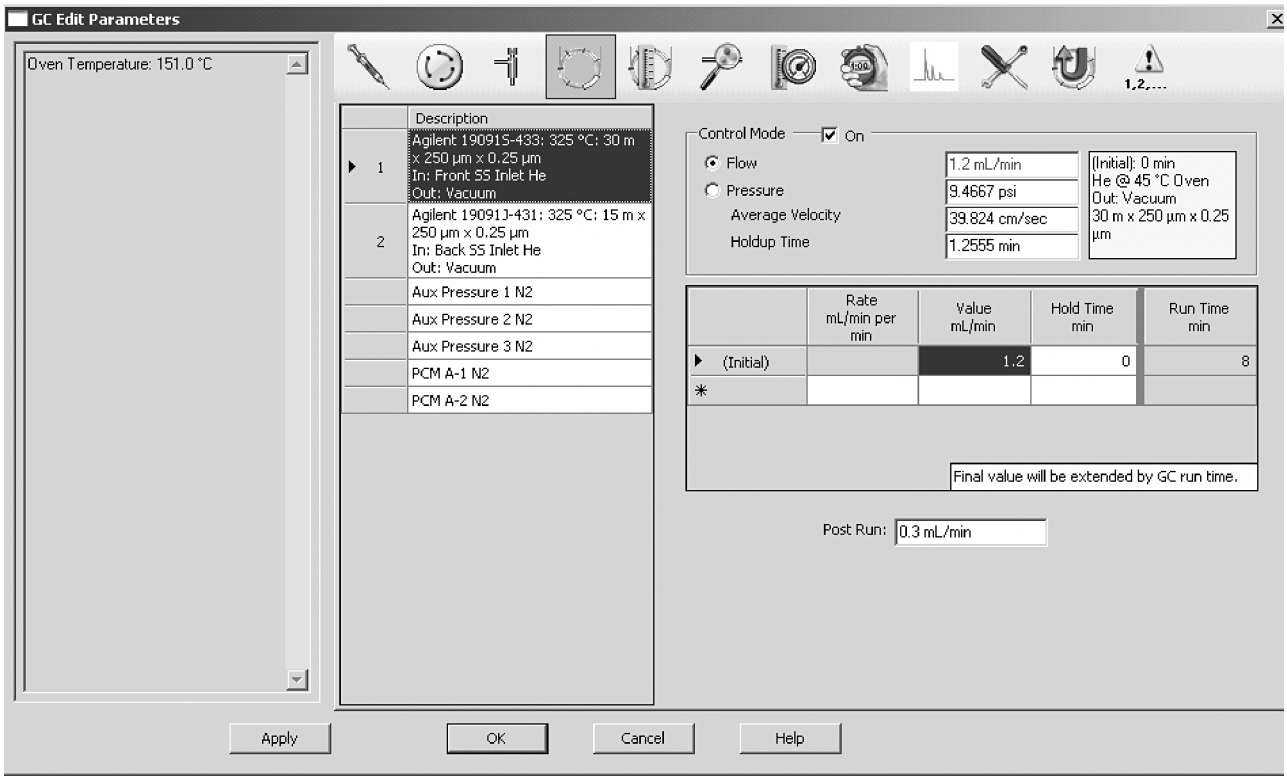



**Figure 9B (middle panel). Adding backflushing in Post Run: front column (column #1) parameters.**

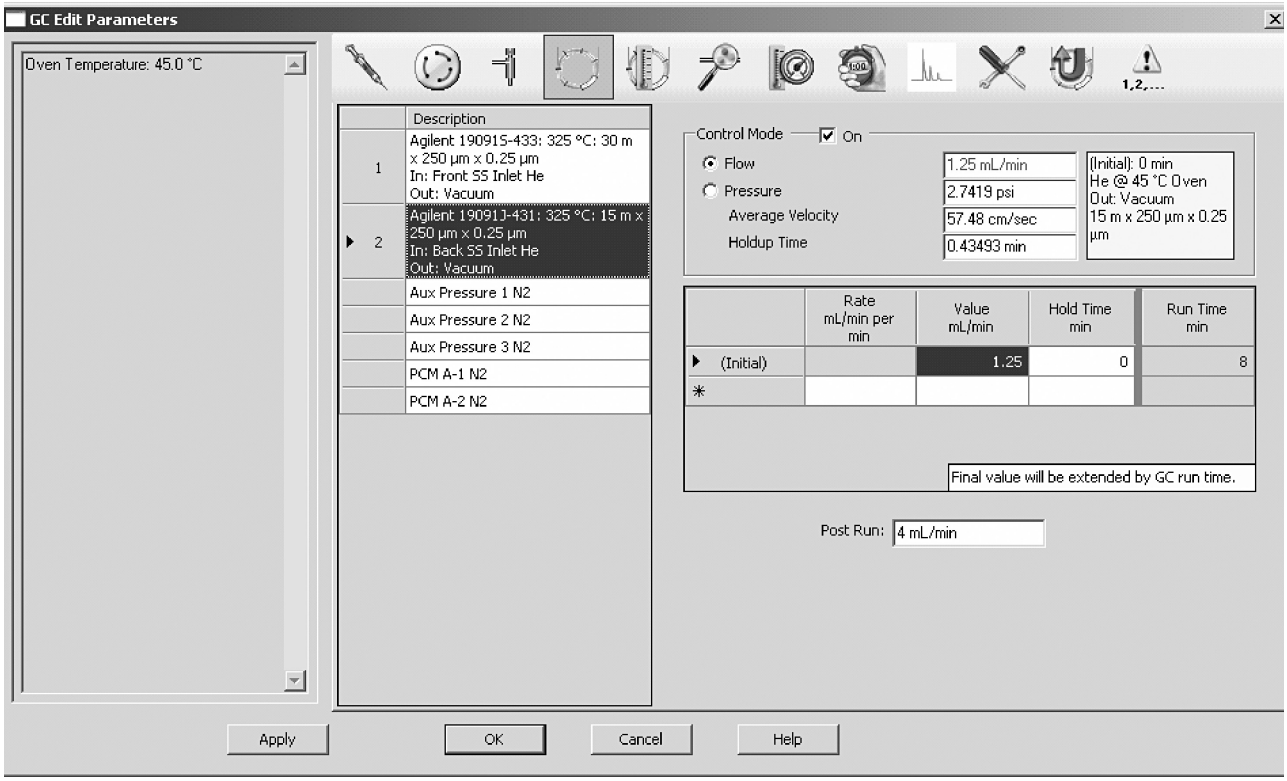



**Figure 9C (lower panel). Adding backflushing in Post Run: back column (column #2) parameters.**

This backflow also prevents fines from the column cutting from entering the column. Make the necessary service and reattach and reload the analytical method.

If a completely new 15-m column (#1) is installed, it can be conditioned *in situ* by setting up the backflow condition with the oven at the conditioning column temperature.

#### Advanced Techniques: Concurrent Backflushing

If the fastest possible total analytical time is the highest priority, one will realize that backflush can begin earlier than the elution of the last component. In other words, backflushing can occur during the analytical acquisition, thereby increasing productivity. After the last compound of interest has passed the CFT tee and entered the back 15-m column, the pressure or flow through the earlier 15-m column can be dropped and compounds will cease moving forward and actually begin to retreat. When the last compound elutes, then the flow in the back column can be raised to complete backflushing. This is demonstrated in Figure 10.

The calculations are also very simple. To calculate when the flow (pressure) in the front column (column #1) is to be reduced, simply subtract the Holdup Time (Figure 9C) from the last compound's

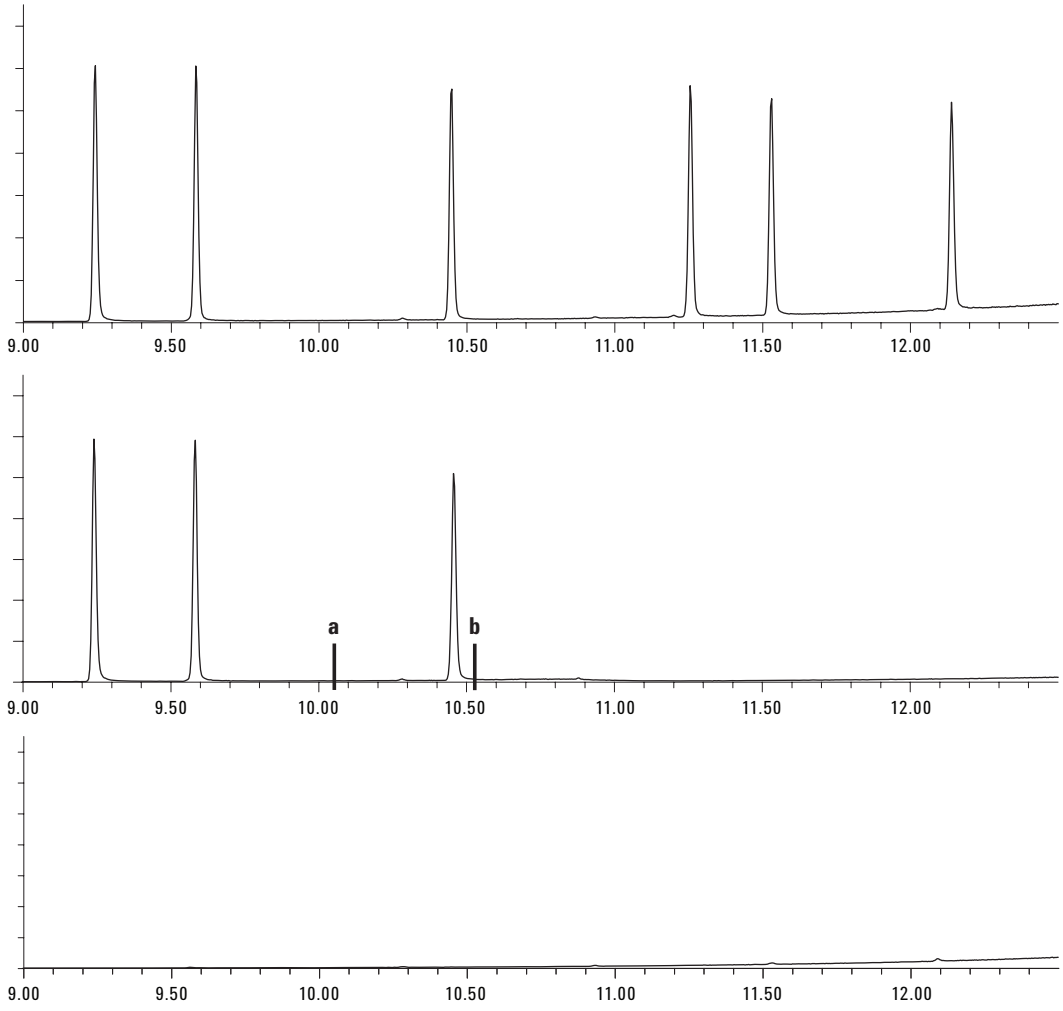



**Figure 10. Example of backflushing with flow or pressure control. Upper panel: RTIC of original six-component standard. The third peak is considered the last analyte and the fourth peak the beginning of the late-eluting interferences. Middle panel: RTIC of the same standard with backflushing beginning at 10.1 min (a), where the first 15-m column (column #1) flow is dropped and at (b) where column #2 flow is increased to 4 mL/min. Note that the last analyte is retained but the late eluters never enter the MSD. Lower panel: solvent blank run without backflush after the backflush method which shows no carryover.**

elution time. After this last compound has eluted, go into Post Run and set the second 15-m column (#2) flow to 4-mL/min (or the pumping system maximum) with the front column (#1) pressure remaining low and the oven at the final programmed temperature. This can best be accomplished in ramped flow mode or in pressure programming. Do this for two to three column volumes and test with a sample followed by a solvent blank to see if this is sufficient. Experimentation with particular samples will enable setting these requirements more efficiently.

## Conclusions

### Alternative Configurations

The CFT is very rich and allows many possible arrangements; these are only a few suggestions or alternatives. The CFT tee used here can be replaced by a purged two-way splitter with one channel plugged (G3180-61500) or even the QuickSwap itself can be moved back from the MSD interface and suspended in the oven.

However, the best CFT tee device appears to be the new Purged Ultimate Union (G3186-60580), Figure 11. As the name describes, this is essentially a union with a gas purging line, making it a very low dead volume tee. It occupies very little space and can be suspended from the column cage, the oven wall, or through the upper GC wall. Preliminary tests of this Purged Ultimate Union using DDT and Endrin have shown very little breakdown. Chromatographic behavior is also very good.

Similarly, the carrier control need not be the back injection port split/splitless module; a Pressure Control Module (PCM) or EPC module can be used. Of the two, the Pressure Control Module may be more convenient.

Most importantly, the CFT tee position itself does not need to be exactly in the middle. The best arrangements can be considered on the basis of selection against components and the rapidity of backflushing. In other words, rapid backflushing suggests a shorter upstream column #1. So another arrangement is at the two-thirds mark or a 10-m column, then the CFT tee, and then a 20-m column to create a 30-m analytical column. Here

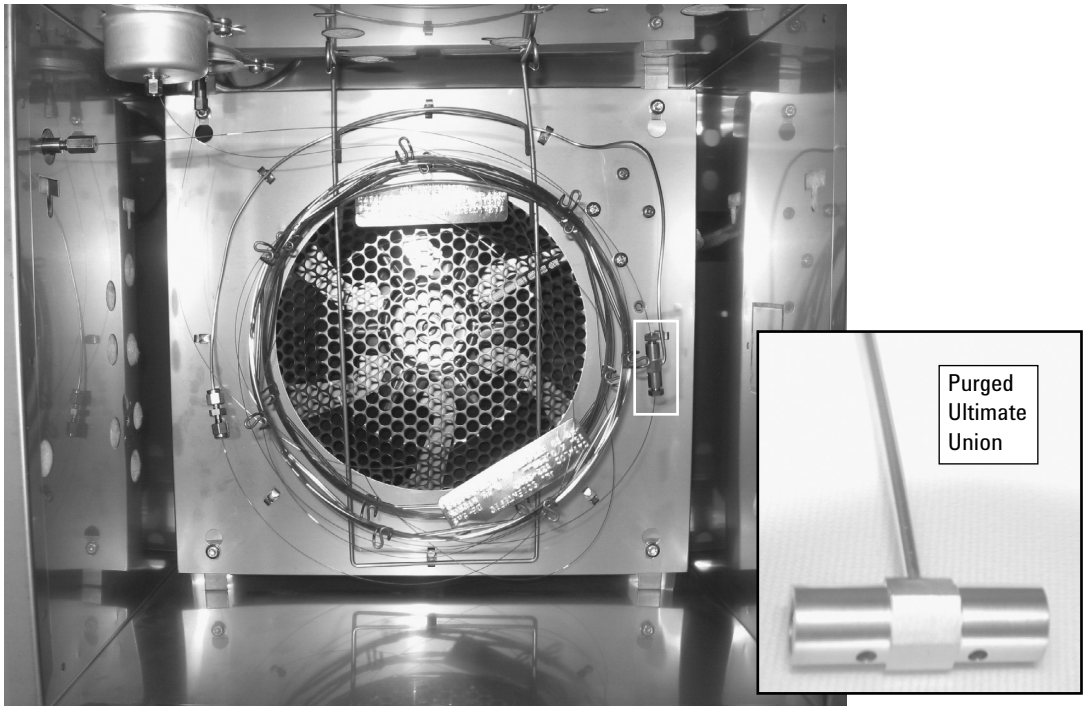



**Figure 11. Purged Ultimate Union.**

backflushing would be nearly 10 times faster than the arrangement with QuickSwap and more than twice as fast as the 15-m column for the same pressure. This would be the best arrangement for the MSD with a diffusion pump. Also, in terms of analytical time, this approach would provide even higher efficiency since 10 column volumes could be flushed in about 2 minutes. If backflushing begins before the analytical run ends (as shown in Advance Techniques and in Figure 10), then in many cases the Post-Run time would be very short or entirely unnecessary, yet still provide sufficient backflushing. This would further reduce total cycle times.

The joined columns need not match in many aspects. For example, a 0.32-mm id may be the first column and a 0.25-mm id the second column. In this situation it will be better to have the columns configured and described as they actually exist in the 7890A. For example, column #1 inlet is the splitless port and the outlet is the PCM module A; column #2 inlet is the PCM module A and the outlet is the MSD. Considerations of capacity, resolution, robustness, etc., can be entertained in several innovative ways to enhance productivity and data quality.

This solution can also be implemented on the Agilent 6890 GC. Of course, the PCT tee configuration is not confined to the Agilent GC/MS detector, but is suitable for other detection schemes as well.

Future software releases will contain a key command that will allow more functionality and greater ease of use: it will allow the user to apply the IGNORE READY = TRUE condition to the EPC device controlling the CFT tee. This will prevent the pressure pulse or other flow conditions from producing a “not ready” condition for the instrument.



## References

1. The 5975C Series GC/MSD, Agilent Technologies publication 5989-7827EN
2. Frank David and Matthew S. Klee, “Analysis of Suspected Flavor and Fragrance Allergens in Cosmetics Using the 7890A GC with Column Backflush,” Agilent Technologies publication 5989-6460EN
3. Frank David and Matthew S. Klee, “GC/MS Analysis of PCBs in Waste Oil Using the Backflush Capability of Agilent QuickSwap Accessory,” Agilent Technologies publication 5989-7601EN

(These references are available in the Literature Library at www.chem.agilent.com.)

## Acknowledgements

The author is very grateful to Bruce Quimby, Wes Norman, and Matthew Klee for several informative and encouraging discussions. Also, a special thanks to Wes Norman for providing superb CFT devices tailored to the needs of the GC/MSD and an advance example of the Purged Ultimate Union.

## For More Information

For more information on our products and services, visit our Web site at www.agilent.com/chem.

Agilent shall not be liable for errors contained herein or for incidental or consequential damages in connection with the furnishing, performance, or use of this material.

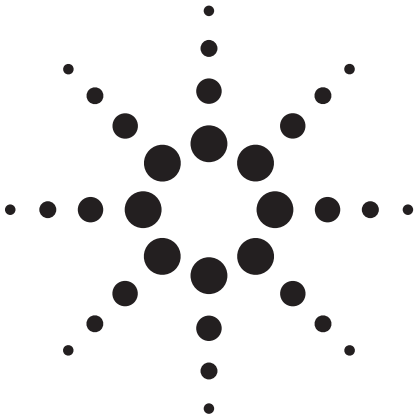
Information, descriptions, and specifications in this publication are subject to change without notice.

© Agilent Technologies, Inc. 2008

Printed in the USA
June 24, 2008
5989-8664EN

Agilent Technologies

# Low-Pressure Retention Time Locking with the 7890A GC



## Application

**HPI**


## Authors

Russell Kinghorn and Courtney Milner
BST, 41 Greenaway Street
Bulleen, VIC 3105
Australia

Matthew S. Klee
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808
USA



## Abstract

**Retention time locking was introduced over a decade ago with the Agilent 6890 gas chromatograph. The next generation of GC from Agilent – the 7890A – has enhanced and extended the functionality previously available, including a new electronic pneumatic control system capable of pressure control to the third decimal place. This application demonstrates the ability of the EPC system to be used for retention time locking at low pressures, in this case with a 320-µm column on a 5975 GC-MS.**


## Introduction

The introduction of retention time locking (RTL) with the 6890 GC gave users a new way of improving productivity by eliminating the need to constantly update compound retention time data whenever a column was trimmed or replaced. It also allowed the same methods to be run on multiple systems with the same retention times. The introduction of eMethods further enhanced the portability of these methods.

Most RTL-based methods were established on GC/MS systems, where a typical head pressure for a 30 m × 0.25 µm id column is > 10 psi. With setability to two decimal places, four-digit pressure setpoints for such columns (for example, 11.54 psig) result in excellent inter-instrument and intra-instrument RTL precision.

Amongst the many optimization tasks in GC method development is deciding on the best column dimension to select. This can realistically only be determined with full knowledge of the sample characteristics and analysis goals (that is, components of interest, complexity, detection limits required, and the matrix of the sample). Larger diameter columns have the advantages of ruggedness and sample capacity over smaller dimension columns. The larger the diameter of the column, the less pressure is needed to establish optimal flows. However, for the most precise RTL, one needs the ability to set pressure very precisely. With two-decimal-place precision at low pressures (for example, 1.28 psig), locking a system to a target retention time is less precise for column dimensions, such as 0.32-µm columns.

The 7890 GC's fifth generation EPC provides excellent low-pressure control, and with third-decimal-place control of the pressure, providing the precision demanded for RTL at low pressures. This application explores the suitability of the 7890 for RTL at low pressures with a 320-µm column and uses a translation of the method described in Agilent Technologies publication 5989-6569EN, "Reliable transfer of existing Agilent 6890/5973GC/MSD methods to the new 7890/5975 GC/MSD."

## Experimental

The method used for this example was translated using the Agilent Method Translator software to convert to a 250-µm id column method to a 320-µm method. A series of standards was run from 10 ppb to 5 ppm to illustrate the performance of the method, followed by trimming approximately 45 cm off the column and relocking the method.

**Table 1. Method Conditions**

| | |
|---|---|
| Column | HP-5 25 m × 0.32 µm × 0.52 µm<br>p/n 19091J-112 |
| Carrier gas | Helium, constant pressure mode –<br>nominal 3.761 psi |
| RTL peak | Pyrene at 11.112 min |
| Split/splitless inlet | 300 °C, pulsed splitless 7 psi for 0.3 in,<br>50 mL/min purge at 0.75 min |
| Oven | 55 °C (1.1 min) to 320 °C (6.5 min) at<br>22.88 °C/min |
| Sample | 1-µL injection, PAH 0.01 to 5 ppm<br>concentration range |
| MSD | Scan 45–400 u<br>Samples = $2^2$<br>Autotune EM + 200V<br>Source = 230 °C<br>Quad = 150 °C<br>Transfer line = 280 °C |

## Results and Discussion

The test sample with 16 polynuclear aromatic hydrocarbons (PAHs) was chosen for this example as it covers a wide range of physical properties and provides several challenging separations. This requires the retention time precision to be as tight as possible to ensure correct identification of the peaks of interest, and relocking must be effective or peak identification will fail on the different column.

Samples were injected in triplicate to measure the retention time precision of the locked method at different concentrations from 0.01 to 5 ppm. Table 2 shows the performance metrics for the retention time and also the correlation co-efficient for the analysis.

The initial retention times in Table 2 were achieved at a head pressure of 3.761 psig. This setpoint was determined from the retention time calibration and relocking process to be appropriate to achieve the target retention time of 11.112 min for the locking compound pyrene. Table 3 shows the calibration data from the RTL runs.

**Table 3. RTL Data**

| Run | Pressure (psi)* | Retention time (mins) | Deviation (seconds) |
|---|---|---|---|
| RTLOCK1.D | 3.01 | 11.212 | 6.018 |
| RTLOCK2.D | 3.38 | 11.166 | 3.264 |
| RTLOCK3.D | 3.76 | 11.112 | 0.000 |
| RTLOCK4.D | 4.14 | 11.070 | –2.508 |
| RTLOCK5.D | 4.51 | 11.020 | –5.508 |
| Maximum deviation | | 6.018 seconds | |
| Correlation co-efficient | | 0.999 | |

* Even though pressure setpoints used for RTL calibration need only be to be to two decimal places, the ability to precisely set locking pressures based on the calibration curve requires setability to the third decimal place.

Figure 1 shows the calibration curves corresponding to the linearity metrics summarized in Table 2.

**Table 2. Retention Time Precision of Low-Pressure RTL**

| | Average retention time | RSD (%) | Calibration linearity ($r^2$) | RT before relocking | Relocked RT | ΔRT when relocked |
|---|---|---|---|---|---|---|
| Naphthalene | 5.884 | 0.055 | 0.997 | 5.809 | 5.876 | 0.008 |
| Acenapthylene | 7.753 | 0.045 | 0.997 | 7.676 | 7.746 | 0.007 |
| Acenaphthene | 7.968 | 0.044 | 0.997 | 7.893 | 7.959 | 0.009 |
| Fluorene | 8.562 | 0.048 | 0.997 | 8.486 | 8.556 | 0.006 |
| Phenanthrene | 9.702 | 0.089 | 0.997 | 9.621 | 9.692 | 0.010 |
| Anthracene | 9.752 | 0.044 | 0.996 | 9.671 | 9.746 | 0.006 |
| Fluoranthene | 11.119 | 0.031 | 0.996 | 11.041 | 11.116 | 0.003 |
| Pyrene | 11.388 | 0.090 | 0.996 | 11.308 | 11.383 | 0.005 |
| Chrysene | 12.808 | 0.094 | 0.996 | 12.719 | 12.799 | 0.009 |
| Benz[a]anthracene | 12.855 | 0.046 | 0.997 | 12.774 | 12.849 | 0.006 |
| Benz[b]fluoranthene | 14.285 | 0.045 | 0.995 | 14.168 | 14.277 | 0.008 |
| Benz[k]fluoranthene | 14.314 | 0.069 | 0.995 | 14.201 | 14.306 | 0.008 |
| Benz[a]pyrene | 14.815 | 0.035 | 0.996 | 14.686 | 14.807 | 0.008 |
| Indeno[1,2,3-cd]pyrene | 17.088 | 0.051 | 0.996 | 16.890 | 17.074 | 0.014 |
| Dibenz[a,h]anthracene | 17.098 | 0.066 | 0.995 | 16.903 | 17.082 | 0.016 |
| Benzo[ghi]perylene | 17.725 | 0.089 | 0.997 | 17.508 | 17.709 | 0.016 |

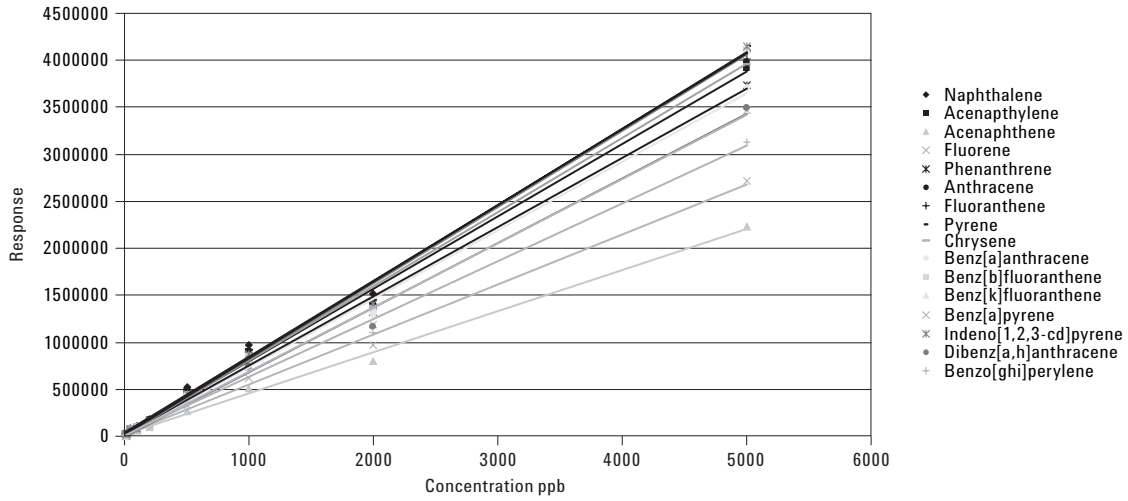



**Figure 1. GCMS calibration curves for 16 PAHs using 320-µm id column with low-head pressure.**

Figure 2 presents overlayed chromatograms for the replicate sample injections, showing the excellent precision of the replicate injections summarized in Table 2.

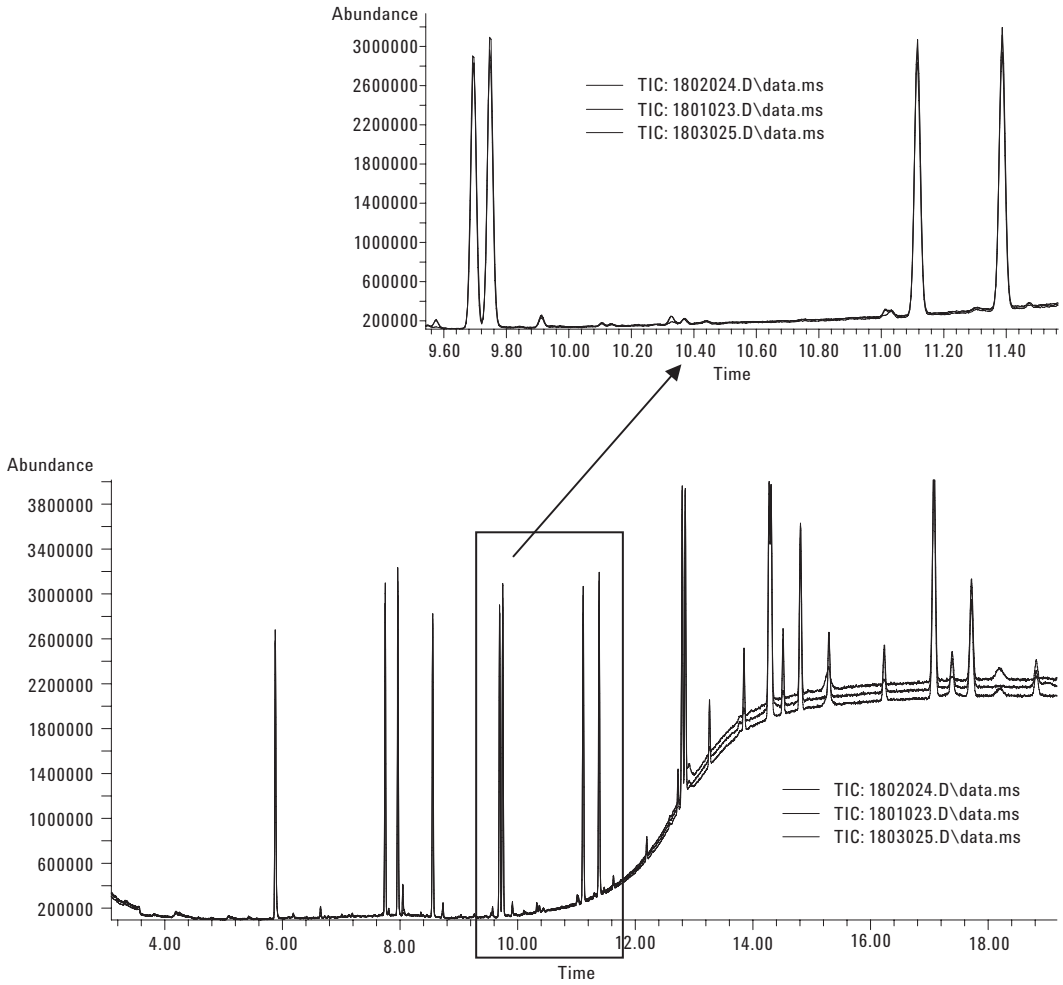



**Figure 2. Overlay of three replicate injections of 2 ppm standard prior to column maintenance and relocking, including a zoomed area of the four peaks from phenanthrene to pyrene.**

A 45-cm length of column was removed from the column to simulate typical maintenance that may be performed on a column during routine use. The method was then relocked and the sample re-run to check the efficacy of relocking at low pressure. The relocked method had a resulting pressure of 3.168 psig. The change in locking pressure can, over time, provide guidance as to the extent at which column trimming can be undertaken with the need for full re-locking of the method.

Figure 3 shows an overlay of the before and after column trimming and the extent of the retention time change. Figure 4 presents an overlay of chromatograms, one of the originals and one after column trimming and relocking. The last two columns of Table 2 compare the relocked retention times of target compounds to the originals.

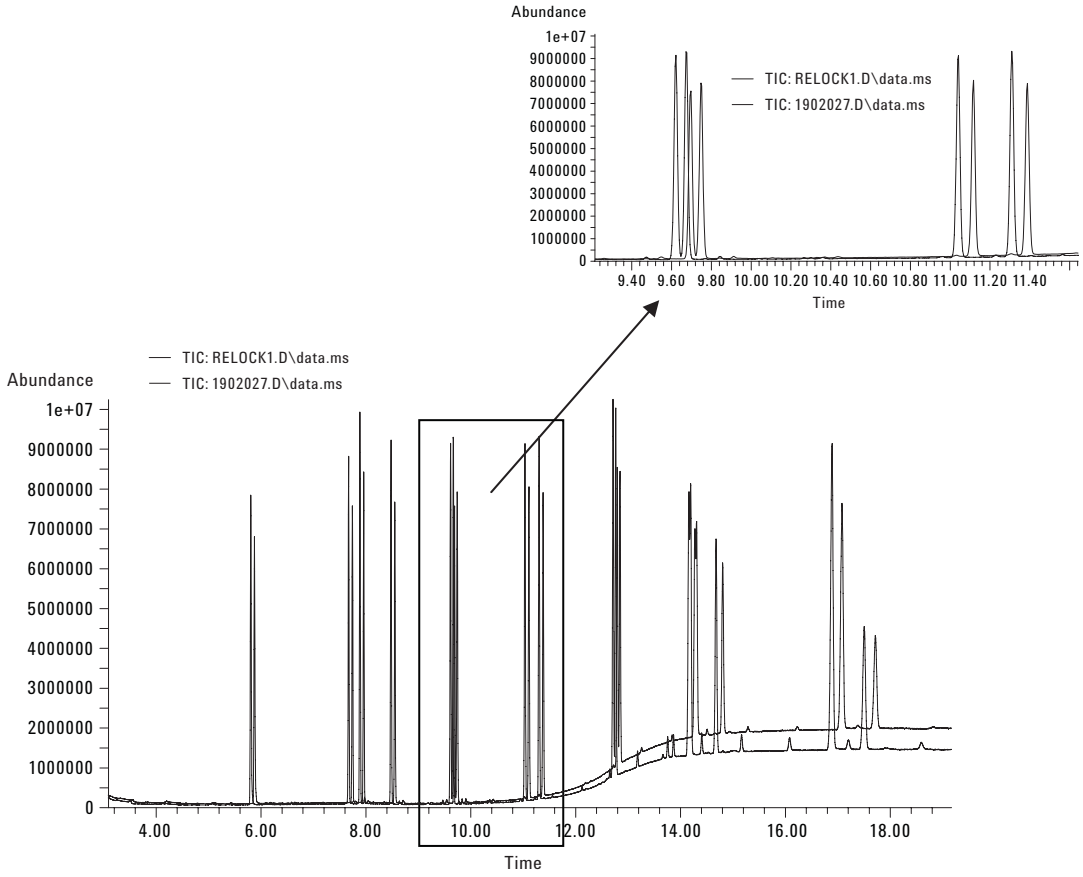



**Figure 3. Overlay of injection before and after column maintenance showing the extent of retention time variation, including a zoomed area of the four peaks from phenanthrene to pyrene**

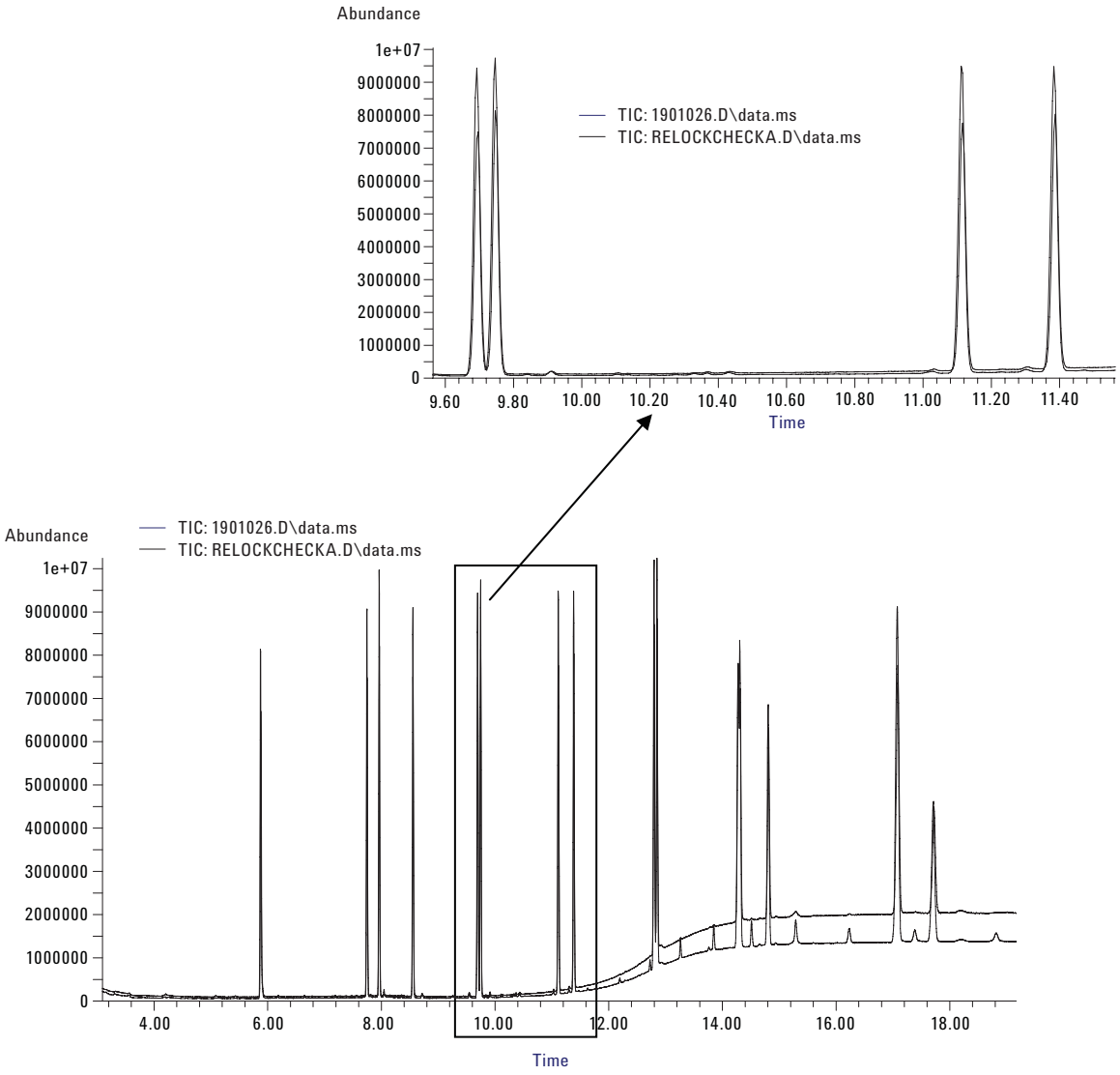



**Figure 4. Overlay of injections before and after column maintenance and relocking, including a zoomed area of the four peaks from phenanthrene to pyrene.**

## Conclusions

This application demonstrates the ability of the 7890 GC system to perform RTL at low pressures (sub 5 psi), such as those experienced when using a 320-µm column in a GC-MS system. The average retention time variation before and after column maintenance for a 16-PAH mixture is less than 0.5 sec, providing high confidence in peak assignments, even with critical separations.

## For More Information

For more information on our products and services, visit our Web site at www.agilent.com/chem.

**www.agilent.com/chem**

Agilent shall not be liable for errors contained herein or for incidental or consequential damages in connection with the furnishing, performance, or use of this material.

Information, descriptions, and specifications in this publication are subject to change without notice.

© Agilent Technologies, Inc. 2008

Printed in the USA
April 8, 2008
5989-8366EN

**Agilent Technologies**

# Precise Time-Scaling of Gas Chromatographic Methods Using Method Translation and Retention Time Locking

Application

Gas Chromatography

May 1998


## Authors

B. D. Quimby, L. M. Blumberg,
M. S. Klee, and P. L. Wylie
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808-1610
USA



## Abstract

**Complete development of a gas chromatographic method often involves a significant amount of effort. Once a method is completed, retention time locking (RTL) can be used to implement the method and to obtain the same retention times on multiple systems. This application note describes how to use method translation combined with RTL to implement precise time-scaled versions of a method on multiple instrument types. This allows the original method to be re-used with minimal effort, while optimizing the method for a given sample type or instrument setup. In this way, the utility of the original method is extended greatly, increasing the payback on the investment in its development and optimizing its use for specific analyses. In this note, the Agilent RTL Pesticide Library method is used as an example. The steps involved in precise time-scaling of the method to different speeds, detectors, and columns are presented.**



## Key Words

Pesticides, GC, GC-AED, retention time locking, RTL, method translation, scalable RT libraries


## Introduction

Interest in the analysis of pesticide residues has been increasing recently, in part due to the discovery that some of these compounds act as endocrine disrupters. Agilent Technologies has responded to the need for rapid, accurate, and comprehensive screening analysis for pesticides by developing a method to screen for 567 pesticides and suspected endocrine disrupters. The method uses element- selective detection and a retention time locked library of retention times to find and identify pesticides in a sample.[1]

In the method, sample extracts are run with element-selective detection using a prescribed set of chromatographic conditions and with the column retention time locked to the retention times in a table. If any peaks containing heteroatoms are observed, the section of the table corresponding to a small time window around the observed peak is searched. The time search results are further sorted using the observed element content of the peak. The combination of time and element content narrows rapidly the possible compounds that could have produced the heteroatom response to a few pesticides.

The element-selective detection is done with either gas chromatography-atomic emission detection (GC-AED), which can screen for all the individual elements found in pesticides, or with a combination of other selective detectors like the electron capture detector (ECD), the nitrogen-phosphorus detector (NPD), the flame photometric detector (FPD), or the electrolytic conductivity detector (ELCD).

The GC-AED technique can also be used to calculate element ratios and to quantitate unknown peaks that are detected because of its equimolar element response factors. The measured element ratios can be used to further distinguish between possible identities of detected heteroatomic compounds, often resulting in a single entry as the likely identity of a given peak. With compound-independent calibration, the amount of the unknown can be calculated using element response factors generated with a different standard compound.

Once the element-selective screen is completed, samples that contain any suspect compounds are run on a GC with mass spectral detection (GC-MS) system that is retention time locked to the pesticide method, thus having the same retention times as the element-selective detectors. Using the possible identities generated from the element screen, the GC-MS data is evaluated to decide which (if any) of the possible identities for suspect peaks is correct. The confirmation process is simplified greatly because the element screen usually yields only a few possibilities and because the retention time in the GC-MS run is accurately known. In practice, extracted ion chromatograms for characteristic ions of each possible compound are used to determine the identity of suspect compounds.

This screening method minimizes false negatives, even in dirty samples, by using element-selectivity and time in the initial screen. With element-selective detection, all compounds containing chlorine, phosphorus, nitrogen, etc. are detected. Even if a detected heteroatomic compound is not in the table, its presence is known, and it can be marked for further GC-MS evaluation. By using GC-MS for confirmation, false positives are also minimized.

The RTL Pesticide Library method is a good example of a method in which a substantial investment of time and material has been made. As with many methods intended for use in multiple laboratories, it would be desirable to be able to scale the method for use in different situations of sample type and instrument setup. Because the method relies on the measured retention times of 567 compounds, it would be impractical to re-measure all the retention times whenever the method is modified, for example, to increase its speed.

Method translation[2–4] is a calculation technique developed at Agilent Technologies that allows a capillary column GC method to be translated to different chromatographic conditions. The technique calculates the required changes in inlet pressure and oven temperature ramp rates and hold times required to maintain peak elution order identical to that of a reference method. In this way, the speed of an analysis can be scaled predictably to accommodate the needs of a specific sample or instrument type.

The inlet pressure calculated for the new version of a method by the method translation software is based on the assumed or nominal dimensions of the column. As such, the calculated inlet pressure will provide a close, but not exact, match to the desired scaled retention times. To match precisely the retention times of the scaled method to the desired scale factor, the new method must be retention time locked. Retention time locking[3] (RTL) is a technique developed by Agilent Technologies whereby the inlet pressure required to match retention times precisely is calculated from a calibration curve of inlet pressure versus retention time.

Using method translation followed by RTL allows a method to be scaled by a precisely known factor. Once the chromatography has been scaled, a retention time table, such as the RTL Pesticide Library, can then be scaled by the same factor, resulting in a new library whose retention times match those of the scaled method precisely.

The steps required to scale the method are:

1. Determine the desired scale factor for the new method.
2. Use the method translation software[4] to calculate the inlet pressure and oven temperature adjustments to obtain the desired scaling of the method. The scale factor is the “speed gain” value reported in the method translation software. Make sure that the new method parameters are consistent with the hardware capabilities of where the new method will be used.
3. Perform the RTL calibration runs for the new method. Alternatively, the method translation software can be used to calculate the RTL calibration points for the new method using those from the original method.
4. Retention time lock the new method using the locking reference standard from the original method. The new method should be locked to the original reference standard retention time divided by the scale factor.
5. Export the retention time table as a text file using the EXPORT function in the RTL SEARCH menu of the RTL ChemStation software.
6. Divide the retention times in the table by the scale factor in a spreadsheet program like Microsoft® Excel™.
7. Re-import the new, scaled table.
8. Run a representative test mixture to validate the scaled method.

Several examples of scaling the HP RTL Pesticide Library are presented below.

## Experimental

All data were collected on Agilent 6890 Series GC systems. All systems were equipped with:

- Electronic pneumatics control (EPC)

- Split/splitless inlet
- Automatic liquid sampler

The GC-AED system also included an Agilent G2350A atomic emission detector with GC-AED ChemStation software (rev B.00.00) for Microsoft® Windows NT®.

The GC-micro-ECD system was controlled by Agilent GC ChemStation software (rev A.05.04). Both the GC-AED and the GC-micro-ECD ChemStations contained RTL software for GC ChemStation (G2080AA) and the Retention Time Locking Pesticide Library for GC ChemStation (G2081AA).

The GC-MS system (G1723A) used consisted of an 6890 Series GC equipped with an Agilent 5973 mass selective detector (MSD). The process for retention time locking the GC-MS system is described in reference 2.

All systems except the micro-ECD instrument used 30 m × 0.25 mm id × 0.25 µm HP-5MS columns (part no. 19091S-433). The Agilent micro-ECD instrument used 10 m × 0.1 mm id × 0.1 µm HP-5 column (part no. 19091J-141).

RTL measurements were made with a solution of dichlorvos, methyl chlorpyrifos, and mirex, each at 10-ppm concentration in acetone. All injections were 1-µL splitless, except for the micro-ECD experiments, which were 1-µL split 100:1. In all methods, inlets were operated at 250 °C and detectors at 300 °C.

Method translation requires inlets to be run in constant pressure mode to obtain precise scaling of retention times. Thus, all methods discussed in the note were run in this mode.

## Results and Discussion

### Locking GC-MS with Other GC Detectors

When using selective GC detectors in conjunction with GC-MS, one problem that is encountered is knowing the relationship between retention times on the selective detector and that of the GC-MS. In GC-MS, the outlet pressure of the column is vacuum, while with most other GC detectors, the outlet pressure of the column is at or near atmospheric pressure. This difference in outlet pressures results in large differences in retention time between GC with MS detection and GC with other detectors. Comparison of GC-FID, a general detector, with GC-MS is reasonably straightforward, because the total ion chromatogram (TIC) of the GC-MS system has similar response to the FID. Retention times on the GC-MS system corresponding to those on the GC-FID can be determined by looking for similar patterns of response. With selective detectors, this is much more difficult because the response patterns from selective detectors usually do not resemble the TIC. For this reason, matching the retention times of selective detectors precisely with the GC-MS system simplifies data analysis greatly.

In this first example of scaling the RTL Pesticide Library, the method will be scaled from the GC-AED method to the GC-MS method. In this case, the desired scale factor is exactly 1, that is, the GC-MS retention times are desired to be exactly the same as those of the GC-AED. The first step is to use the method translation software to determine the GC conditions to use for GC-MS.

Figure 1 shows the method translation software. The original method conditions for the GC-AED pesticide method are entered in the column labeled “Original Method.” The column dimensions, carrier gas type, inlet pressure, outlet pressure, ambient pressure, and oven temperature program are entered here. Note that the inlet pressure is in psi (gauge), while the outlet pressure and ambient pressure are psi (absolute). The original method here is being used on a GC-AED system, so the outlet pressure is entered as atmospheric pressure plus 1.5 psi, the operating pressure of the GC-AED.

The “Criterion” parameter is set to “None,” which allows the user to select a specific value of “speed gain” by adjusting the value of hold-up time for the translated method (see figure 1). In the column labeled “Translated Method,” the parameters of column dimensions, carrier gas type, outlet pressure, and ambient pressure for the GC-MS method are entered. Note that the inlet pressure and oven program are not entered; they are calculated by the program. To set the speed gain to a desired value, take the calculated value of hold-up time in the first column (0.996060 minute) and divide it by the scale factor. Because in this case the desired scale factor (“speed gain”) is 1, the same hold-up time for both the GC-AED and the GC-MS methods is required. Clicking the radio button next to the hold-up time in the “Translated Method” column will do this automatically.

The method translation indicates that to obtain the same retention times on the GC-MS system as on the GC-AED, use all the same method parameters

except inlet pressure. Instead of using 27.6 psi as is used on the GC-AED, method translation calculates that 17.93 psi on the GC-MS system will result in matching retention times. As mentioned above, this inlet pressure is calculated on the assumed dimensions of the column in the GC-MS system. To get the retention times to match precisely, RTL[3] is used.

To retention time lock the GC-MS method to the GC-AED method in this example, it is necessary to construct an RTL calibration file for the GC-MS system. Construction of this file only needs to be done once. All subsequent users of the GC-MS method will then be able to use this calibration file for a similarly configured GC-MS instrument.

The RTL calibration file is constructed by running five calibration runs of the target compound, in this case methyl chlorpyrifos, at five different inlet pressures. The runs are made at conditions identical to the nominal method except that four of the runs are made at different pressures. The pressures used are typically:

- Target pressure – 20%
- Target pressure – 10%
- Target pressure (nominal method pressure)
- Target pressure + 10%
- Target pressure + 20%

The retention time of the target compound is determined for each run. The resulting set of five pressures and corresponding retention times is then entered in the RTL calibration dialog box for the method and saved with the method.

To lock the method on the GC-MS setup, the target compound is run at

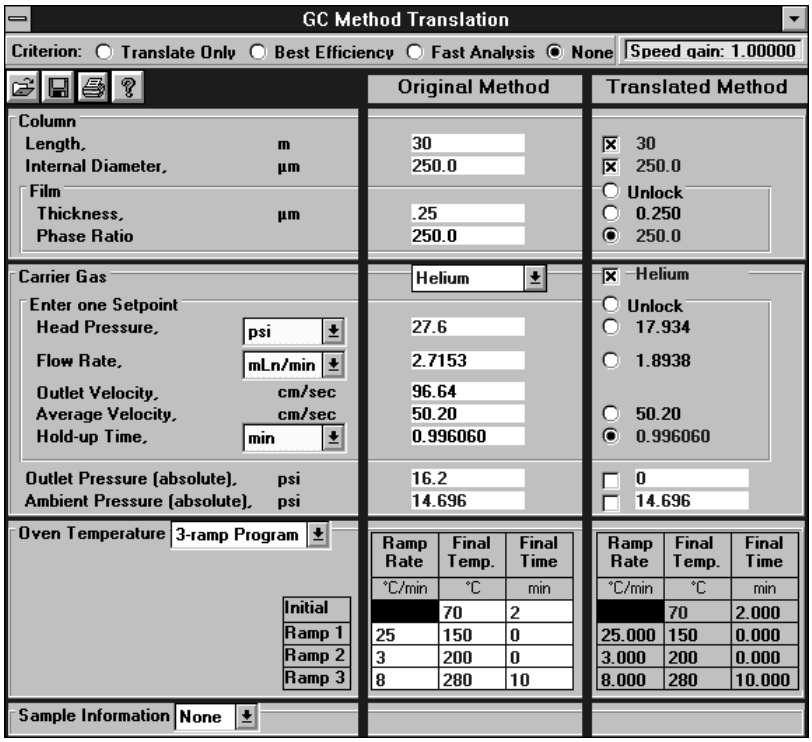

GC Method Translation
Criterion: Translate Only Best Efficiency Fast Analysis None
Speed gain: 1.00000
Original Method
Translated Method
Column
Length, m 30 30
Internal Diameter, µm 250.0 250.0
Film
Thickness, µm .25 Unlock
Phase Ratio 250.0 0.250 250.0
Carrier Gas Helium Helium
Enter one Setpoint
Head Pressure, psi 27.6 Unlock 17.934
Flow Rate, mLn/min 2.7153 1.8938
Outlet Velocity, cm/sec 96.64
Average Velocity, cm/sec 50.20 50.20
Hold-up Time, min 0.996060 0.996060
Outlet Pressure (absolute), psi 16.2 0
Ambient Pressure (absolute), psi 14.696 14.696
Oven Temperature 3-ramp Program
Ramp Rate °C/min Final Temp. °C Final Time min
Initial 70 2 70 2.000
Ramp 1 25 150 0 25.000 150 0.000
Ramp 2 3 200 0 3.000 200 0.000
Ramp 3 8 280 10 8.000 280 10.000
Sample Information None

**Figure 1. Method translation software showing scaling HP RTL Pesticide Method from GC-AED conditions to GC-MS with a scale factor of 1.**

the nominal method pressure, and the retention time is observed. The pressure and resulting retention time are then entered into the "(Re)Lock New Column" menu item of the RTL software to calculate the correct pressure for obtaining locked retention times.

Normally, the RTL calibration for a new method is determined by actually making the five calibration runs. In the current example, methyl chlorpyrifos would be run at:

- 17.93 psi – 20% = 14.34 psi
- 17.93 psi – 10% = 16.14 psi
- 17.93 psi (nominal method pressure)
- 17.93 psi + 10% = 19.72 psi
- 17.93 psi + 20% = 21.56 psi

However, because the new GC-MS method is scaled from an existing GC-AED method that already has RTL calibration data, method translation can be used to calculate the new RTL calibration points. This is useful when you want to try a scaled method rapidly and save the time required in making the five runs. (Note: For methods that will be used extensively, the five-runs approach may provide a somewhat better calibration. It is recommended that for these methods, the standard calibration be performed.)

To calculate the five RTL calibration pairs of pressure and retention time for the GC-MS method from those of the GC-AED method:

- Take the inlet pressure used for each original GC-AED RTL calibration run, and enter it into the method translation software for the inlet pressure of the original method. Make sure the hold-up times are locked, giving a "speed gain" of 1.

- The inlet pressure calculated in the "Translated Method" column will now change to a new value, corresponding to the pressure that would be obtained if the calibration run were made on a GC-MS system. This pressure is used with the retention time obtained for the corresponding GC-AED calibration run as a calibration point for the GC-MS method.

When all five points have been calculated in this way, they are entered into the RTL calibration dialog box for the GC-MS method and saved with the method. Table 1 lists the original RTL calibration pressures and times with the calculated pressures and times for the GC-MS method.

To test the accuracy of using a predicted RTL calibration file for GC-MS, a real calibration set was measured on the GC-MS system. The data is shown in the first two columns of table 2. (Note: The calibration points are spaced ~ 5% apart in pressure instead of the typical 10%.) A GC-MS RTL calibration file was constructed with these measured points. For each point, the locking pressure required to lock the method was calculated and is shown in column 3 of table 2.

The locking pressure is the pressure determined by the RTL software that would make methyl chlorpyifos have a retention time of 16.596 minutes. This is determined by entering the pressure and retention time for each point into the "(Re)Lock New Column" menu item of the RTL software. If the calibration is done correctly, the locking pressures determined from each point should be very similar, as they are in column 3 of table 2.

Column 4 of table 2 shows the locking pressures for the same set of runs but determined using the GC-MS RTL calibration points calculated using method translation. The calculated data provide locking pressures that agree well with those based on measured data. The range in locking pressures pressure is only from 17.72 to 17.75 psi. This range of 0.03 psi corresponds to only about a 0.006-minute range in the retention time of methyl chlorpyrifos.

Figure 2 shows the locked chromatograms from a three-component mixture run on GC-AED and GC-MS systems. As can be seen, the retention times are well matched between the two methods.

The RTL Pesticide Library contains the retention times of the 567 pesticides measured with GC-FID. The values measured with the FID would be the same observed with any detector that is operated at or near atmospheric pressure. Because retention time matching is critical in this application, the retention times for all the compounds in the table were also measured on the GC-MS system after scaling as described here. Figure 3 is a plot of the difference between the retention times measured on the GC-FID and the GC-MS systems. The plot shows the retention times match well within ± 0.1 minute out to 30 minutes. A few compounds at the end deviate outside this window, with one compound 0.2-minute different. The

**Table 1. RTL Calibration Points from Original GC-AED Method and Calculated Points for GC-MS**

| GC-AED RTL Calibration | | GC-MS RTL Calibration | |
|---|---|---|---|
| Pressure (psi) | Ret Time (min) | Calculated Pressure (psi) | Calculated Ret Time (min) |
| 33.1 | 15.346 | 24.27 | 15.346 |
| 30.4 | 15.919 | 21.18 | 15.919 |
| 27.6 | 16.578 | 17.934 | 16.578 |
| 24.8 | 17.338 | 14.654 | 17.338 |
| 22.1 | 18.242 | 11.449 | 18.242 |

**Table 2. Comparison of Locking Pressures Calculated Using Measured and Predicted GC-MS RTL Calibration Data**

| GC-MS Locking Runs | | Locking Pressures | |
|---|---|---|---|
| Measured GC-MS RTL Cal Points | | Using Measured RTL Cal Points | Using Calculated RTL Cal Points |
| Pressure ( psi) | Ret Time (min) | Pressure (psi) | Pressure (psi) |
| 20 | 16.127 | 17.73 | 17.75 |
| 19 | 16.326 | 17.72 | 17.73 |
| 18 | 16.536 | 17.72 | 17.72 |
| 17 | 16.760 | 17.74 | 17.74 |
| 16 | 16.988 | 17.72 | 17.74 |

deviation is clearly largest in the isothermal hold region, which starts at 31.87 minutes. This effect is seen with GC-MS, but not with scaling to other atmospheric pressure detectors. While the cause is not yet clearly understood, it appears related to the vacuum outlet pressure of the GC-MS column. Although this level of matching is very good, the table includes both the GC-FID and GC-MS retention times so that smaller time windows can be used in searching unknowns.

### Locking GC-AED with Other GC Detectors

When the method translation step is done to scale the GC-AED method to other atmospheric pressure detectors, the only different parameter to enter is the outlet pressure. The outlet pressure for the GC-AED method is 16.2 psi and that for the others is 14.696 psi. The method translation calculates that the nominal GC-AED inlet pressure of 27.6 psi would be changed to 26.29 psi for the other atmospheric detectors. This difference (<5%) is so small that it can be neglected, because corrections in this range are compensated easily by the retention time locking step. Thus, the method conditions and RTL calibration points used with GC-AED are interchangeable with FID, NPD, ECD, FPD, and other atmospheric detector methods.

Note that this would not always be the case. If for example, a method is being scaled that uses a very low inlet pressure, the 1.5-psi difference in outlet pressure could become significant. It is best to check the method with method translation and see if the inlet pressure will change by >10%. If it does, it would be advisable to collect (or translate) a new RTL calibration centered around the translated nominal inlet pressure.

### Gaining Speed in the Same Instrument Setup

In the analysis of pesticide residues in food, there are usually only a few compounds encountered in any one sample. Because the screening method uses selective detectors, it makes sense to consider trading speed for chromatographic resolution. Selective detectors respond to only those compounds containing a specific heteroatom(s), and the chromatography only needs to resolve those compounds from each other, not from every other compound in the matrix. This approach can save a significant amount of analysis time.

In this example of scaling the RTL Pesticide Library, the method will be increased in speed at the expense of chromatographic resolution. The first consideration is by what factor to increase the speed. The method translation software is useful for determining this. A candidate speed gain, in this example threefold, is entered into the method translation software. The resulting inlet pressure and oven temperature ramp rates are then inspected to see if the instrument on

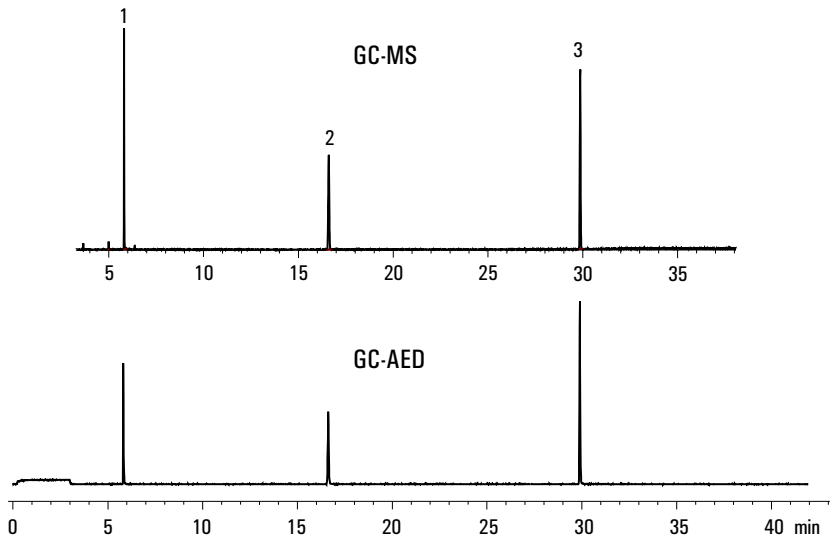



**Figure 2. GC-AED chlorine and GC-MS TIC chromatograms of three-component locking mixture. Peak identifications: 1. dichlorvos, 2. methyl chlorpyrifos, 3. mirex.**

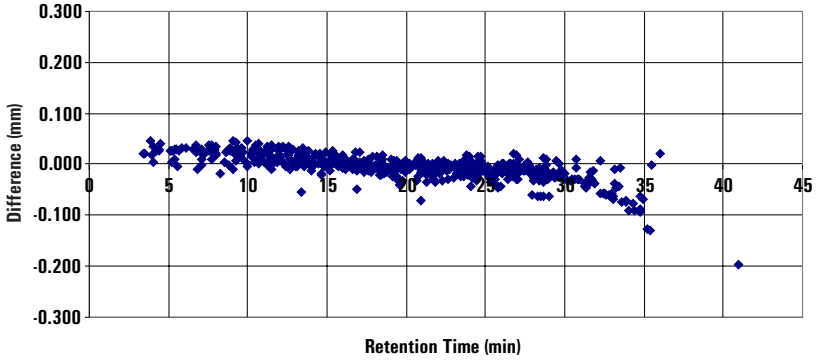



**Figure 3. Difference plot of GC-MS and GC-FID retention times in RTL Pesticide Library.**

which the new method will be run is compatible with those parameters.

Figure 4 shows the method translation software with the data entered for a speed gain of 3. Note that columns for "Original Method" and "Translated Method" are set up as in the previous example with two exceptions. Because the scaling is from GC-AED to GC-AED, the outlet pressure in both columns is entered as 16.2 psi. The second and most significant difference is the holdup time. The desired "speed gain" is 3.

To set the speed gain, the calculated value of hold-up time in the first column (0.996060 minute) is divided by exactly 3. This value (0.33202 minute) is entered for the hold-up time in the second column. This will force the speed gain to exactly 3.

The inlet pressure and oven temperature ramp for the new threefold speed method are now calculated. The calculated inlet pressure is 87.862 psi, which is compatible with the EPC module on the current system (maximum 100 psi). Note that the helium source supplying the GC must be capable of reaching 100 psi of helium. An optional 150-psi EPC module is available for the HP 6890 GC to provide additional inlet pressure, if necessary.

The oven temperature program calculated for the new method has the first ramp listed as 75 °C/min. This ramp rate is compatible with the 240-V oven option on the current instrument but would not work with a 120-V oven, which is limited to about 50 °C/min in this temperature range. With a 120-V oven, the speed gain would be limited to about 2.

The next step is to calculate the RTL calibration points from the original GC-AED method. This is done by the same process as shown in the GC-MS scaling above. In this case, when one of the original method RTL calibration pressures is entered, the resulting holdup time must be divided by 3 and entered for the holdup time in the "Translated Method" column. This will force the "speed gain" back to 3. The resulting inlet pressure is then paired with the retention time of the corresponding original GC-AED calibration run, but divided by 3 as a calibration point for the new method.

Table 3 shows the RTL calibration points from the original GC-AED method and calculated points for the threefold speed gain (3×) method.

When the calibration data is entered into the RTL calibration dialog box, the target time for methyl chlorpyrifos is entered as 5.532 minutes, which is 16.596 minutes divided by 3.

Table 4 compares the locking pressures determined with measured and with calculated RTL calibration points. As in the above GC-MS example, the range of the locking pressures from the calculated data is only 0.11 psi (87.88 to 87.99), which corresponds to ~ 0.003 minute.

Figure 5 compares the chromatograms of the RTL locking mixture from both the original and the 3× scaled methods. Note that while the chromatographic resolution is reduced, the speed is increased by a factor of 3.

Figure 6 shows a plot of the difference between the RTL Pesticide Library retention times, divided by 3, and those of the 3× method. The data were taken with a 36-component subset of the library. The plot shows the retention times match well within ± 0.05 minute for all compounds, even

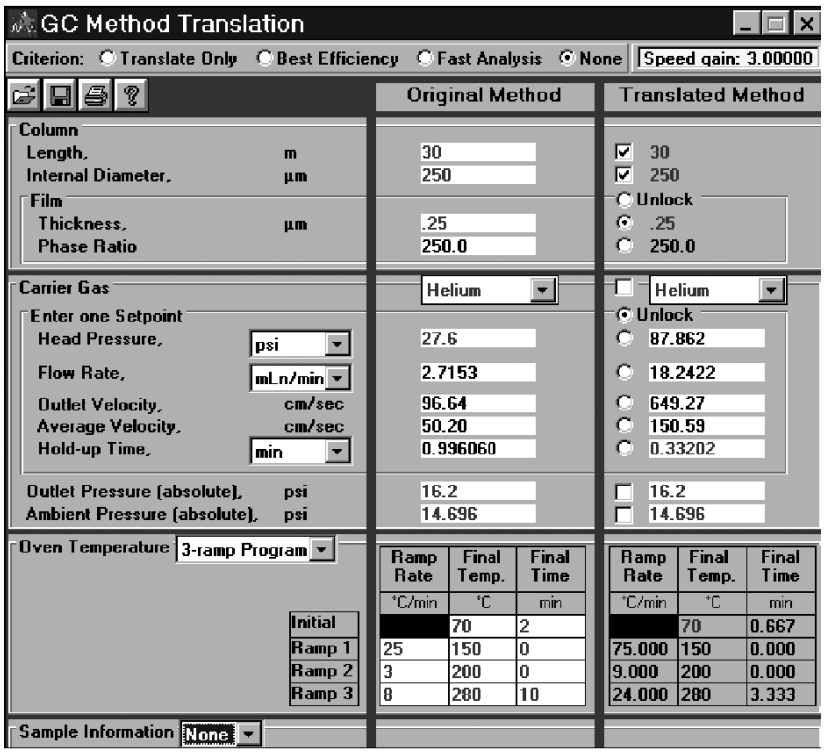

GC Method Translation
Criterion: Translate Only Best Efficiency Fast Analysis None
Speed gain: 3.00000
Original Method
Translated Method
Column
Length, m 30 30
Internal Diameter, µm 250 250
Film
Thickness, µm .25 Unlock .25
Phase Ratio 250.0 250.0
Carrier Gas Helium Helium
Enter one Setpoint
Head Pressure, psi 27.6 Unlock 87.862
Flow Rate, mL/min 2.7153 18.2422
Outlet Velocity, cm/sec 96.64 649.27
Average Velocity, cm/sec 50.20 150.59
Hold-up Time, min 0.996060 0.33202
Outlet Pressure (absolute), psi 16.2 16.2
Ambient Pressure (absolute), psi 14.696 14.696
Oven Temperature 3-ramp Program
Ramp Rate °C/min Final Temp. °C Final Time min
Initial 70 2 70 0.667
Ramp 1 25 150 0 75.000 150 0.000
Ramp 2 3 200 0 9.000 200 0.000
Ramp 3 8 280 10 24.000 280 3.333
Sample Information None

**Figure 4. Method translation software showing scaling RTL Pesticide method scaled to threefold faster method.**

those in the 3.3-minute hold time at the end of the run.

## Gaining Speed with a Small-Bore Column

In the previous example, speed was gained at the expense of resolution. In this example, speed will be gained while maintaining most of the resolution but sacrificing capacity. This is done by scaling the original method to a 0.1-mm id column.

In scaling to columns of a different diameter, there are two important considerations that must be obeyed to obtain precise matching to a library or reference method. The first is that the stationary phase composition must be the same as that used in the original method. The second is that the phase ratio of the column being scaled to must be the same as that of the reference method. Columns of the same phase ratio have the same ratio of inner diameter to film thickness. Because the reference method was developed on a column with 0.25 mm id × 0.25 µm film thickness, scaling to a 0.1-mm id column will require a 0.1-µm film thickness. A 10-m column of these dimensions was chosen for this example.

The micro-ECD for the 6890 GC is extremely sensitive, with detection limits in the low femtogram range for polyhalogenated pesticides. These detection limits are so low that it is reasonable to consider using split mode for a rapid screening method. Using split mode with a split ratio of 100 still gives a detection limits in the range of a few picograms. The split is also more compatible with the relatively low capacity of the column.

**Table 3. RTL Calibration Points from Original GC-AED Method and Calculated Points for Threefold Speed Gain (3×) Method**

| GC-AED RTL Calibration | | 3x GC-AED RTL Calibration | |
|---|---|---|---|
| Pressure (psi) | Ret Time (min) | Calculated Pressure (psi) | Calculated Ret Time (min) |
| 33.1 | 15.346 | 106.21 | 5.115 |
| 30.4 | 15.919 | 97.23 | 5.306 |
| 27.6 | 16.578 | 87.86 | 5.526 |
| 24.8 | 17.338 | 78.44 | 5.779 |
| 22.1 | 18.242 | 69.31 | 6.081 |

**Table 4. Comparison of Locking Pressures Calculated Using Measured and Predicted 3× GC-AED RTL Calibration Data**

| 3x GC-AED Locking Runs | | Locking Pressures | |
|---|---|---|---|
| Measured 3x GC-AED RTL Cal Points | | Using Measured RTL Cal Points | Using Calculated RTL Cal Points |
| Pressure ( psi) | Ret Time (min) | Pressure (psi) | Pressure (psi) |
| 97 | 5.319 | 87.99 | 87.99 |
| 92 | 5.433 | 87.94 | 87.95 |
| 87 | 5.557 | 87.99 | 87.99 |
| 82 | 5.689 | 87.99 | 87.96 |
| 77 | 5.832 | 87.97 | 87.88 |

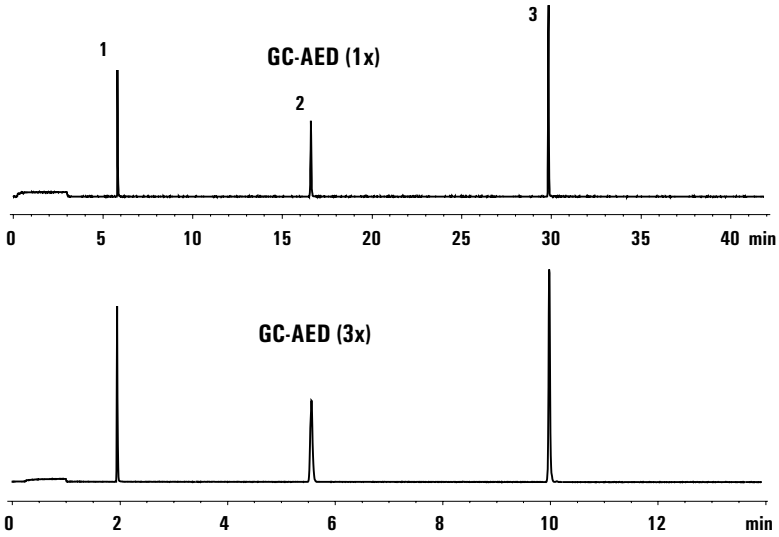



**Figure 5. Chlorine chromatograms from original and 3x GC-AED methods of three-component locking mixture. Peak identifications: 1. dichlorvos, 2. methyl chlorpyrifos, 3. mirex.**

Figure 7 shows the method translation from the GC-AED method to the 0.1-mm id column with a scale factor of 3. A speed gain of 3 was again chosen based on oven and inlet limitations as described above. The same scaling process as used above is followed.

The RTL calibration points for the new 3× 0.1-mm micro-ECD method were both calculated with method translation and measured. Table 5 shows the calculated values.

When the locking pressures from the measured and calculated values were examined, the calculated values provided much poorer predictions of locking pressure than expected. The pressure required to actually lock the column was confirmed to be 65.95 psi, as predicted by the measured RTL calibration data. Method translation had predicted the inlet pressure would be 58.514 psi for an assumed 10-m column length. Because the actual locking pressure was noticeably higher, this suggests that the actual column length was longer and/or the column diameter was smaller and/or the film thickness larger than the assumed values.

As an experiment, it was assumed that the problem was in the assumed length of the column used in calculating the RTL calibration points. The column length entry for the 0.1-mm column was iteratively adjusted until the calculated inlet pressure matched the actual locking pressure, 65.95 psi. This resulted in a calculated column length of 10.5622 m. A new set of calculated RTL calibration points were calculated using 10.5622 m as the length of the 0.1-mm column. The results are shown in table 6.

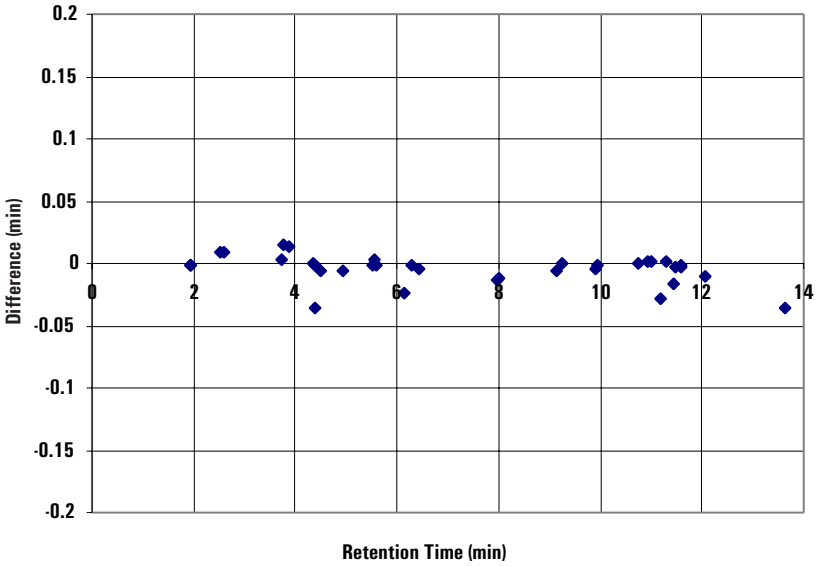



**Figure 6. Difference plot of RTL Pesticide Library (GC-FID) retention times divided by 3 minus 3× GC-AED retention times for 36-compound subset of the library.**

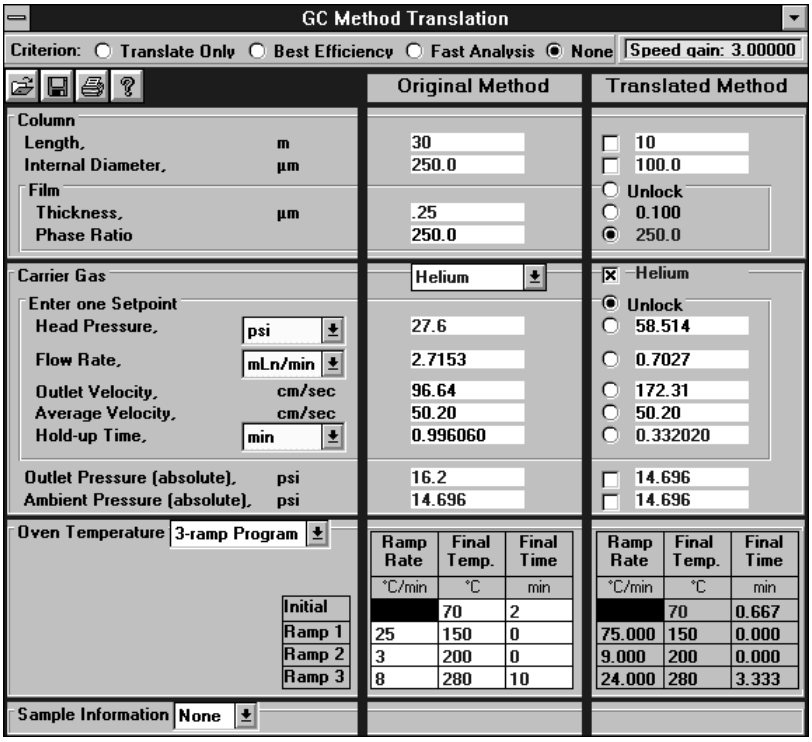

GC Method Translation
Criterion: Translate Only Best Efficiency Fast Analysis None
Speed gain: 3.00000
Original Method
Translated Method
Column
Length, m 30 10
Internal Diameter, µm 250.0 100.0
Film
Thickness, µm .25 Unlock
Phase Ratio 250.0 0.100 250.0
Carrier Gas Helium Helium
Enter one Setpoint Unlock
Head Pressure, psi 27.6 58.514
Flow Rate, mLn/min 2.7153 0.7027
Outlet Velocity, cm/sec 96.64 172.31
Average Velocity, cm/sec 50.20 50.20
Hold-up Time, min 0.996060 0.332020
Outlet Pressure (absolute), psi 16.2 14.696
Ambient Pressure (absolute), psi 14.696 14.696
Oven Temperature 3-ramp Program
Ramp Rate °C/min Final Temp. °C Final Time min
Initial 70 2 70 0.667
Ramp 1 25 150 0 75.000 150 0.000
Ramp 2 3 200 0 9.000 200 0.000
Ramp 3 8 280 10 24.000 280 3.333
Sample Information None

**Figure 7. Method translation software showing scaling RTL pesticide method scaled to a threefold faster method on a 10-m × 0.1-mm id column.**

Table 7 shows a comparison of locking pressures calculated using measured and predicted 3× 0.1-mm id micro-ECD calibration data. The range of locking pressures from the measured data (66.03 to 65.93) only corresponds to a spread in retention times of about 0.004 minute. However, with the data calculated based on a 10-m assumed length, the spread (66.38 to 63.18) is much larger and would correspond to a time range of 0.14 minute. The locking pressures calculated using the 10.5622 value are much more consistent with the measured values. The range in retention times would be ~ 0.03 minute if all the calculated points are used, and if the first value in column 5 is ignored, the range drops to ~ 0.005 minute.

The fact that the agreement in locking pressures is much improved by using 10.56 m instead of 10 m suggests that length is probably the largest contributor to the discrepancy. These results should reinforce the recommendation that if a method is to be used extensively, it is prudent to obtain measured RTL calibration data. It should be noted, however, that even with the RTL calibration from the 10-m assumed length, the worst consequence would be that the RT locking step would need to be repeated an extra time to get a more precise match.

Figure 8 compares the chromatograms of the RTL locking mixture from both the original and the 3 × 0.1-mm id micro-ECD methods.

**Table 5. RTL Calibration Points from Original GC-AED Method and Calculated Points for 3× 0.1-mm id Micro-ECD Method Assuming 10-m Column Length**

| GC-AED RTL Calibration | | 3x Micro-ECD RTL Calibration | |
|---|---|---|---|
| Pressure (psi) | Ret Time (min) | Calculated Pressure (psi) | Calculated Ret Time (min) |
| 33.1 | 15.346 | 71.03 | 5.115 |
| 30.4 | 15.919 | 64.90 | 5.306 |
| 27.6 | 16.578 | 58.51 | 5.526 |
| 24.8 | 17.338 | 52.11 | 5.779 |
| 22.1 | 18.242 | 45.91 | 6.081 |

**Table 6. RTL Calibration Points from Original GC-AED Method and Calculated Points for 3× 0.1-mm id Micro-ECD Method Assuming 10.5622-m Column Length**

| GC-AED RTL Calibration | | 3x Micro-ECD RTL Calibration | |
|---|---|---|---|
| Pressure (psi) | Ret Time (min) | Calculated Pressure (psi) | Calculated Ret Time (min) |
| 33.1 | 15.346 | 80.03 | 5.115 |
| 30.4 | 15.919 | 73.13 | 5.306 |
| 27.6 | 16.578 | 65.95 | 5.526 |
| 24.8 | 17.338 | 58.74 | 5.779 |
| 22.1 | 18.242 | 51.75 | 6.081 |

**Table 7. Comparison of Locking Pressures Calculated Using Measured and Predicted 3× 0.1-mm id Micro-ECD Calibration Data**

| 3x Micro-ECD Locking Runs | | Locking Pressures | | |
|---|---|---|---|---|
| Measured 3x Micro-ECD RTL Cal Points | | Using Measured RTL Cal Points | Using 10-m Calculated RTL Cal Points | Using 10.56-m Calculated RTL Cal Points |
| Pressure (psi) | Ret Time (min) | Pressure (psi) | Pressure (psi) | Pressure (psi) |
| 48.81 | 6.323 | 65.95 | 66.38 | 65.30 |
| 52.66 | 6.041 | 66.03 | 65.77 | 65.85 |
| 58.51 | 5.797 | 65.95 | 65.12 | 65.96 |
| 64.36 | 5.585 | 65.93 | 64.36 | 65.95 |
| 70.22 | 5.396 | 66.00 | 63.18 | 65.90 |

Note that while the most of the chromatographic resolution is preserved, the speed is increased by a factor of 3.

After being locked, the three peaks in the 3× micro-ECD method had retention times of 1.924, 5.533, and 9.963 minutes, respectively. These values are very close to the RTL Pesticide Library retention times for the three compounds divided by 3: 1.932, 5.532, and 9.949. The fact that the largest difference between the scaled table and the 3× micro-ECD method is only 0.014 minute again demonstrates the precision of retention time matching achievable with the scaling technique described here.

## Conclusions

Using method translation combined with retention time locking provides a means of extending the usefulness of existing capillary GC methods. The ability to precisely scale a method to meet the needs of different samples and instrument types greatly reduces the effort required to re-use methods, thus saving time and money.

## References


1. P. L. Wylie and B. D. Quimby, "A Method Used to Screen for 567 Pesticides and Suspected Endocrine Disrupters," Hewlett-Packard Company, Application Note 228-402, Publication 5967-5860E, April 1998.
2. M. Klee and V. Giarrocco, "Predictable Translation of Capillary GC Methods for Fast GC," Hewlett-Packard Company, Application Note 228-373, Publication 5965-7673E, March 1997.
3. V. Giarrocco, B. D. Quimby, and M. S. Klee,"Retention Time Locking: Concepts and Applications," Hewlett-Packard Company, Application Note 228-392, Publication 5966-2469E, December 1997.
4. Capillary Column Method Translator, user contributed software, free download from: www.hp.com/go/mts.

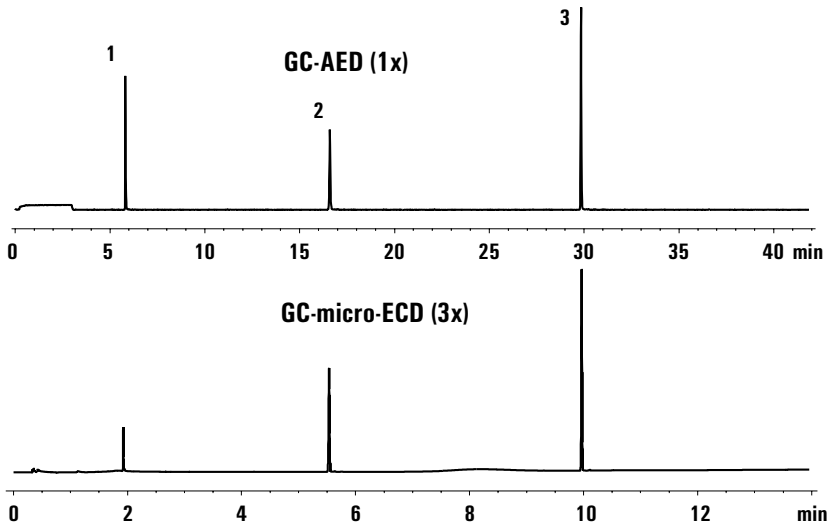




**Figure 8. Chlorine chromatogram from 1× GC-AED method (top) and 3× micro-ECD method (bottom) of three-component locking mixture. Peak identifications: 1. dichlorvos, 2. methyl chlorpyrifos, 3. mirex.**

Agilent shall not be liable for errors contained herein or for incidental or consequential damages in connection with the furnishing, performance, or use of this material.

Information, descriptions, and specifications in this publication are subject to change without notice.

Microsoft® and Windows NT® are U.S. registered trademarks.

Copyright © 2006
Agilent Technologies, Inc.

Printed in the USA 1/2006
5967-5820E

**Agilent Technologies**
Innovating the HP Way

# The 5973N inert MSD: Using Higher Ion Source Temperatures

Application


## Authors

Harry Prest and Charles Thomson
Agilent Technologies, Inc.
5301 Stevens Creek Boulevard
Santa Clara, CA 95052-8059



## Abstract

**The new 5973N inert MSD and ChemStation software (G1701DA) offers the capability of operating the ion source at higher temperatures. This feature, combined with the improved inertness of the source, can provide the user with improvements in analysis, if exploited coherently. This application note provides advice and examples of how to explore the utility of ion source temperature.**


## Introduction

The default ion source temperature of 230 °C is commonly applied in electron impact (EI) ionization on the 5973 MSD platforms. The new Inert Source when used with the new revision of the ChemStation software (rev. DA) allows ion source temperature to be set to a maximum of 300 °C. As with all advances, there are advantages and disadvantages in operating at higher source temperatures. This note will address several general aspects in EI operation.

## Tuning

Figures 1 and 2 show the results for autotuning the Inert Source at the standard 230 °C ion source temperature and the 300 °C temperature limit of the new source (quadrupole temperature 200 °C). The higher temperature for the source produces a perfluorotributylamine (PFTBA) spectrum that shows lower abundances of the higher mass fragments, which is not entirely unexpected. The *m/z* 219 fragment has dropped to an abundance comparable to the *m/z* 69 ion and the ion at *m/z* 502 has dropped about 50%. This is to be expected as the internal energy of the calibrating gas has increased. Note, however, that the isotopic ratios are maintained.

The user should also expect to see a higher background in the higher temperature tunes. A portion of the background will be due to ions associated with column bleed. Bleed, which usually condenses in the source, now is volatized and will appear as an increase in background and baseline.

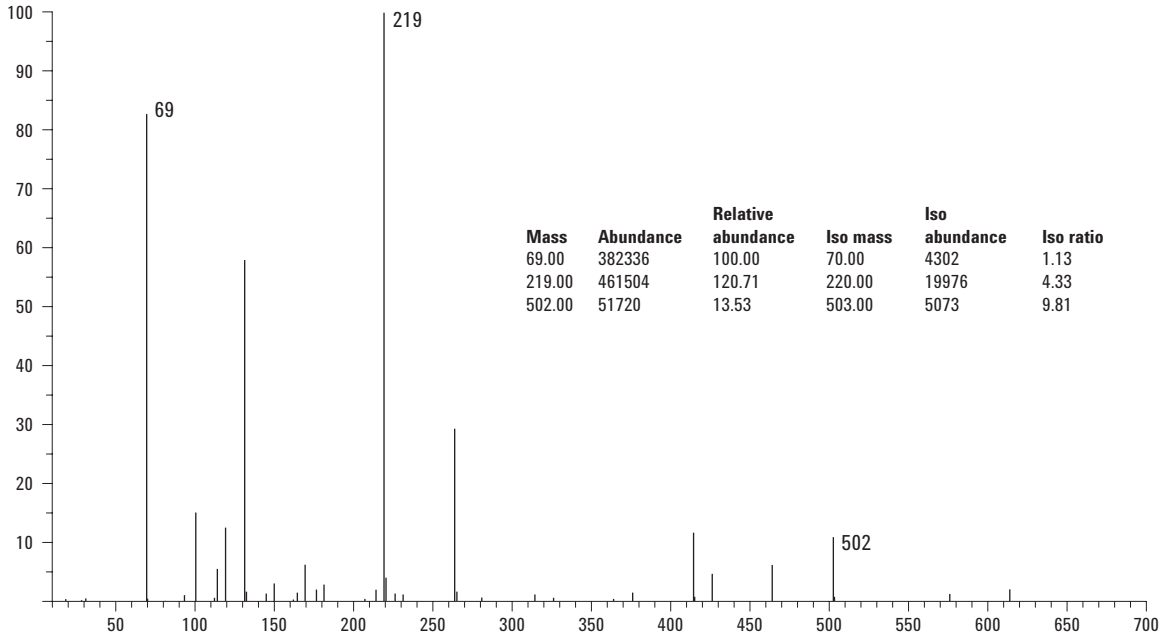



**Figure 1. Autotune results for an ion source temperature of 230 °C.**

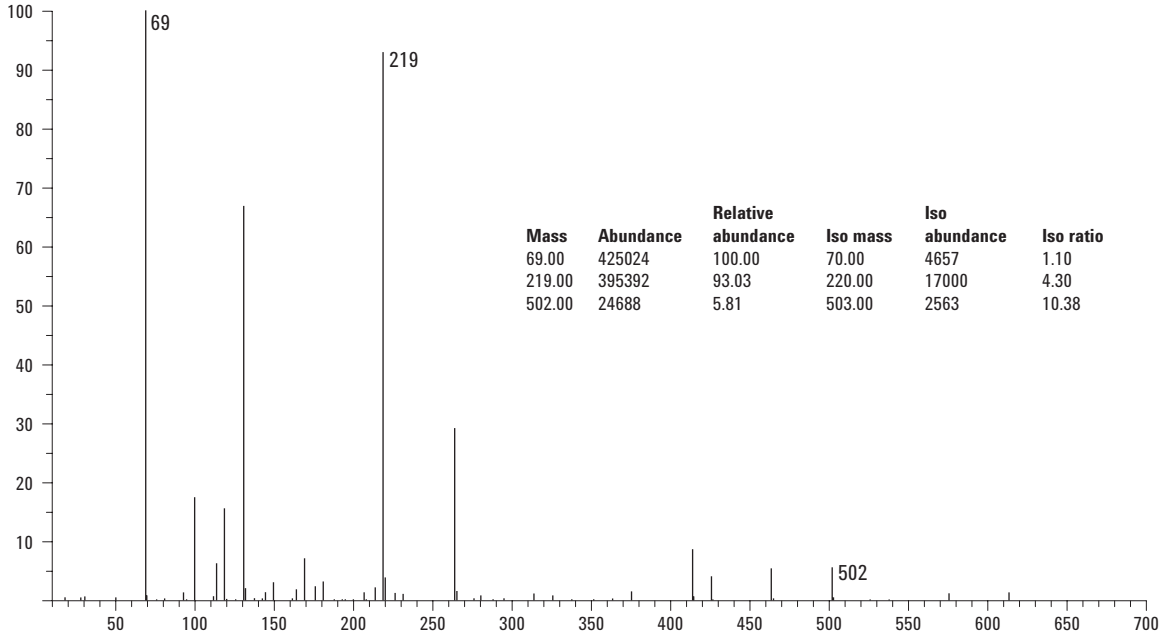



**Figure 2. Autotune results for an ion source temperature of 300 °C.**

## Implications for Analytical Applications

Although the tuning compound showed a spectral change that favored more fragmentation, and all compounds could be expected to be influenced similarly, there are some advantages that can occur for less fragile compounds, especially those that have higher boiling points and are late eluting in GC. Analysis of the class of compounds known as "persistent organic pollutants" (POPs) is likely to benefit from higher source temperatures.

To illustrate the aspects that need to be examined, consider the six polychlorinated biphenyls (PCBs) acquired in full-scan and presented in Figure 3. The overlaid reconstructed total-ion-current chromatograms (RTICCs) suggest that the higher source temperature increases the total response for the later eluting PCBs but produces little enhancement for the early eluters. This could be due to more fragmentation and may not necessarily be useful if the increase in the RTIC is due to lower mass fragments since these lower mass ions are usually compromised by interferences. A calculation of the signal/noise (S/N) for the RTICCs shows that while there is an increase in signal at the source higher temperature, there is also an increase in the background noise and the result is a lower S/N ratio for the higher source temperature.

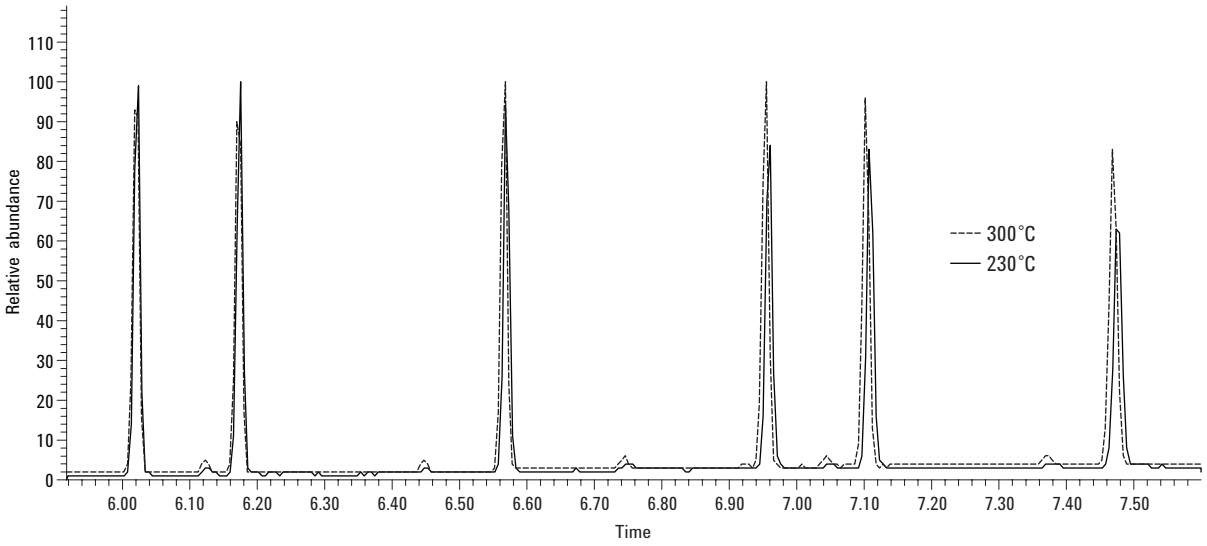



**Figure 3. Overlaid RTICC of six PCBs acquired in full-scan (50–505 amu) at source temperatures of 230 °C and 300 °C. From left to right, or earlier to later, in the chromatogram, the PCBs consist of a $Cl_3$-Biphenyl, $Cl_4$-B, $Cl_5$-B, $Cl_6$-B, another $Cl_6$-B and a $Cl_7$-B.**

Figure 4 shows the same analytes acquired in selected-ion-monitoring mode (SIM) using three ions for each component (M, M+2 or M–2, and M–70). The same trend appears with an enhancement apparent in signal for the later eluting PCBs but little increase for the earlier PCBs. Now, however, the RTIC for the SIM acquisition does show a higher S/N ratio for these later PCBs. As opposed to the full-scan acquisition, the SIM mode acquisition at higher source temperature does increase signal for the ions of interest and, because there was no increase in background, a useful S/N increase was obtained. As always, the guiding principle that an increase in signal is only useful if it exceeds the concomitant increase in background holds. This is clearly illustrated by the third PCB, the pentachlorobiphenyl ($Cl_5$–B). Figure 5 shows the behavior of the signal and background for the two source temperatures for one of the pentachlorobiphenyl confirming ions. The higher source temperature raises the signal and the background for this ion of interest over the lower temperature but fortunately signal increases faster than background. In this case, the background is due to column bleed components and is unavoidable but fortunately not very intense. This may or may not be the case in sample analysis.

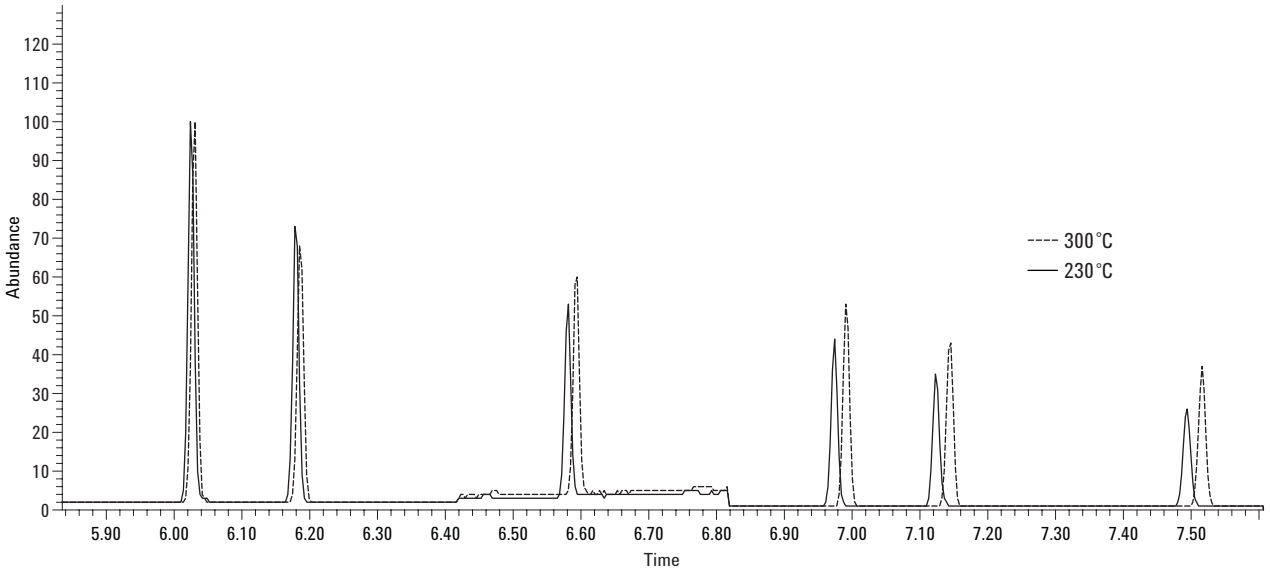



**Figure 4. Overlaid RTICC of six PCBs acquired in SIM at source temperatures of 230 °C and 300 °C. From left to right, or earlier to later, in the chromatogram the PCBs consist of a $Cl_3$-Biphenyl, $Cl_4$-B, $Cl_5$-B, $Cl_6$-B, another $Cl_6$-B and a $Cl_7$-B.**

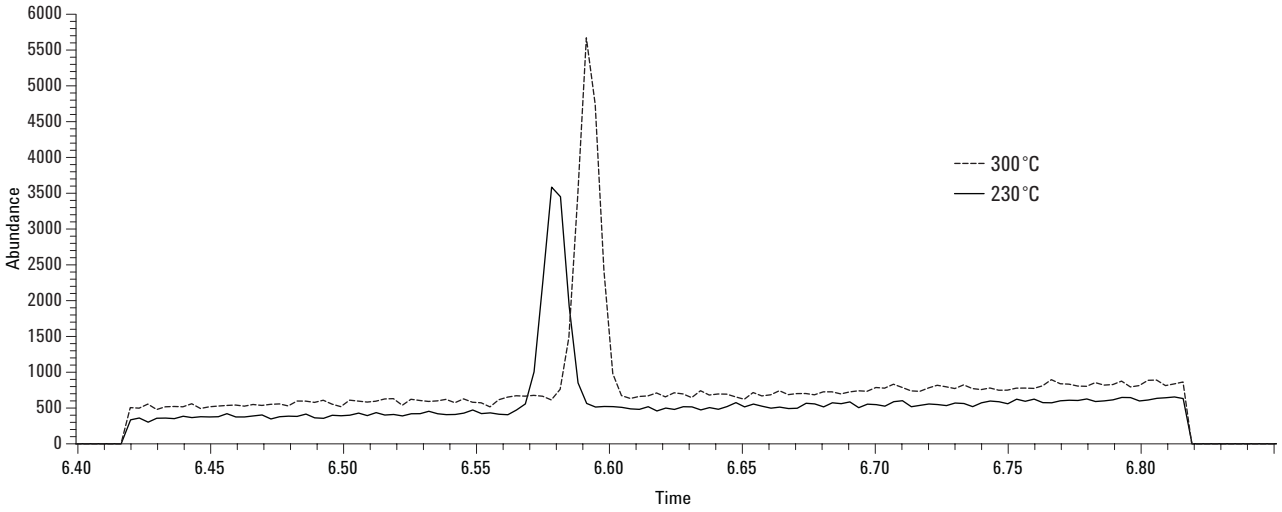



**Figure 5. Overlaid extracted ion-current chromatograms of one ion (M-70) for the pentachlorobiphenyl acquired in SIM at source temperatures of 230 °C and 300 °C.**

The detection limits for many late eluting, "high-boiling" compounds that will improve by implementing higher source temperatures (for example, PAHs, terphenyls, etc.). As an illustration of the enhancement for very "high-boiling" compounds, consider the 6-ring benzenoid hydrocarbon (PAH), coronene (CAS 191-07-1). This compound is difficult to determine due to low response and poor chromatography, although it is present in many sediment samples. Figure 6 shows overlaid RICCs for acquisitions of coronene at 230 °C and 300 °C. Although the peak area is the same, the enhanced Gaussian peak shape achieved at 300 °C improves detection.

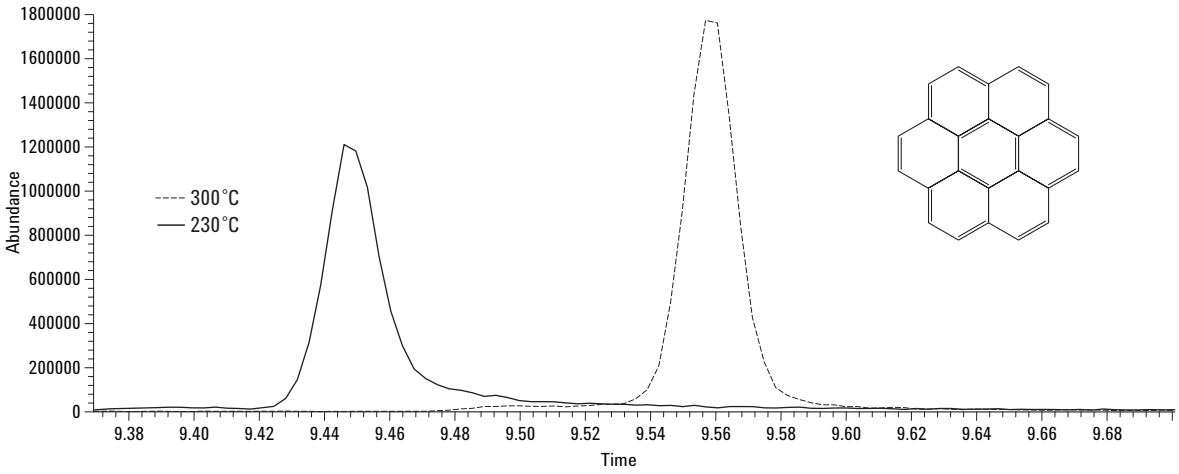



**Figure 6. Overlaid extracted ion-current chromatograms of one ion (*m/z* 300) for coronene acquired in full scan at source temperatures of 230 °C, and 300 °C.**

## Source "Bakeout"

There may be considerable temptation to use the higher source temperature for source “cleaning” by “baking”. In other words, when the user notices a higher background in the source or a reduction in response, the ill-conceived approach of baking the source clean may come to mind. The result will be that “garbage” coating the source will be volatized further into the analyzer; the other lenses will get dirtier, as will the multiplier, etc. “Baking” is not a substitute for mechanical cleaning of the source. However, baking a source after a cleaning is a good approach and a macro that provides this option is given in Table 1. After a source has been cleaned, and the MS system pumped down and checked to be leak free, this macro can be implemented either manually or in a sequence. (Note that the temperature limits in the tune file need to be altered to 300 and 200 for source and quadrupole, respectively). Manually the bakeout is called from the command line in TOP by –

```
macro "bake.mac"  <enter>
bake 2  <enter>
```

The “2” calls for a 2 hour bakeout, and which can be set to anytime the user requires.

Copy the lines in Table 1 into Notepad and save the file as BAKE.MAC in the MSDCHEM\MSEXE directory. The “!” indicates a comment (line) which is not executed. Note that the temperature limits, which reside in the tune file, must be edited to allow the higher settings.

**Table 1. ChemStation Macro for Baking the Source and Quadrupole After Source Maintenance**

```
name Bake
! this macro sets the source and quad temps to their maximum and holds for a set period
parameter hours def 6                  ! default setting is 6 hours -this is customizable
msinsctl "mstemp QUAD, , , 200"        ! sets the quad temperature to bake at 200C
synchronize
msinsctl "mstemp SOURCE, , , 300"      ! sets the source temperature to bake at 300C
synchronize
SLEEP hours*60*60                      ! bakes for set period
msinsctl "mstemp QUAD, , , 150"        ! sets the quad temperature to operating temp at 150C
synchronize
msinsctl "mstemp SOURCE, , , 230"      ! sets the source temperature to operating temp at 230C
synchronize
return
```

Usually a source cleaning is executed at the end of the working day, and the system pumped down overnight for operation the next day. In this case, a "pumpdown sequence" is useful. After the system is confirmed to be leak-tight, this sequence is loaded and executed which bakes the source and quad overnight, then executes an Autotune, and then makes a few injections of a checkout standard to confirm system performance. In this way, the analyst returns the next day to review data about the system prior to beginning new analyses. An example of this is given in Figure 7.

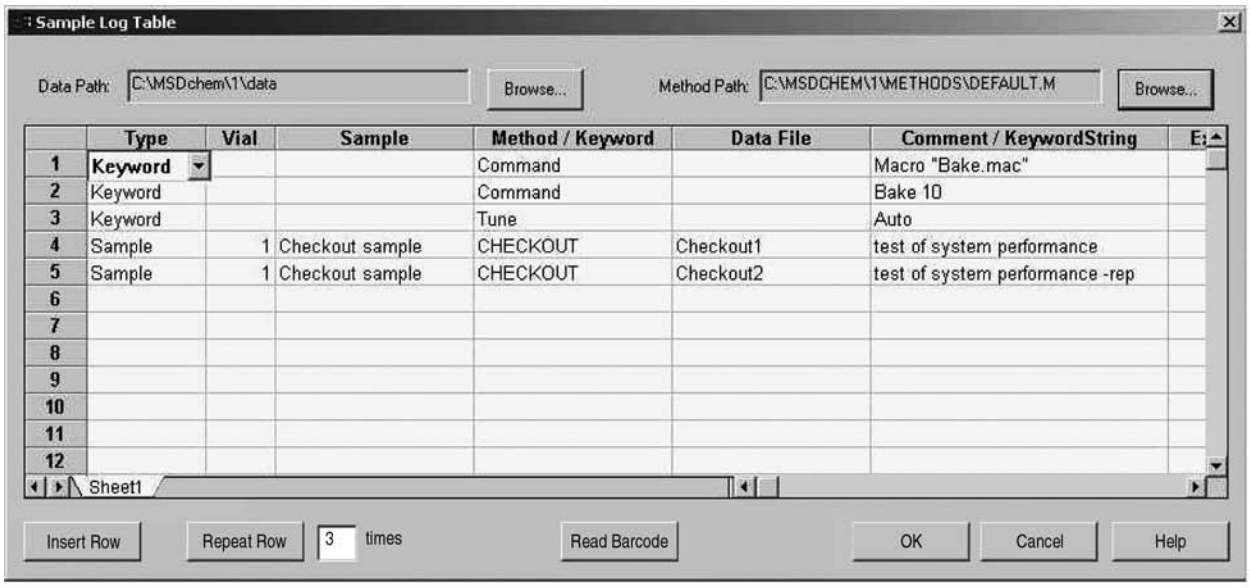



**Figure 7. Pumpdown sequence table using source bakeout.**

Line 1 Loads the Bake macro. Line 2 sets the bake time to 10 hours. After the bake, (Line 3) an autotune is executed. Lines 4 and 5 run the system performance method, CHECKOUT.M, on the system checkout standard. Note: after the system has been cleaned and leak-checked, the CHECKOUT.M method should be loaded, THEN this sequence should be run!

## Conclusions

The increased source temperature limit available on the 5973N inert MSD can provide improved detection limits for common, late-eluting, recalcitrant compounds such as the POPs when properly applied. A requirement, that must be explored, is that the higher source temperatures do not increase compound fragmentation or reduce the intensity of the (useful) higher mass ions. These improvements are most likely to be realized in SIM acquisitions where the increased background that must result from higher source temperatures is not as likely to affect the signal.

This application note also describes a programmed bake-out of the source and quadrupole that can be automatically implemented after source cleaning. This bake-out provides a rapid lowering of the air-water background and can be used within the sequence table as part of the instrument performance checkout.

## For More Information

For more information on our products and services, visit our Web site at www.agilent.com/chem.

Agilent shall not be liable for errors contained herein or for incidental or consequential damages in connection with the furnishing, performance, or use of this material.

Information, descriptions, and specifications in this publication are subject to change without notice.

© Agilent Technologies, Inc. 2004

Printed in the USA
February 10, 2004
5989-0678EN

Agilent Technologies

# Combined EI and CI Using a Single Source

## Technical Overview

Chris Sandy

*Agilent Technologies*

## Introduction

The Agilent 5973x gas chromatograph/mass selective detectors (GC/MSDs) come with sources optimized for electron ionization (EI) and chemical ionization (CI). However, there are occasions where another ionization mode is desired without changing sources. This note demonstrates the capability of acquiring high-quality EI spectra with the CI source.

## Data Acquisition

An Agilent 5973 inert MSD with a CI source was set up for the experiments. The following process was used to tune the MS:

1. Perform the CI autotune at the normal methane reagent gas flow rate (typically at a mass flow controller (MFC) setting of 20%).
2. Reduce the CI flow to 2%.
3. Set the emission current to 250 µa.
4. In Manual Tune, ramp the repeller from 0–5 volts for the mass 69 ion.
5. Set the repeller voltage to the maximum value.
6. Turn off the CI gas.
7. Save tune file.
8. Associate tune file with method.

Data was acquired in positive CI (PCI) and EI modes. Figure 1 shows the CI and EI total ion chromatograms using the CI source. The major and minor peaks are easily comparable in the two chromatograms.

Figure 2 shows the CI spectrum for Hexadecanolide (MW = 254) with the expected adduct ions for methane. Note the relatively large response for the 255 ion. As expected, there is little fragmentation due to the soft ionization.

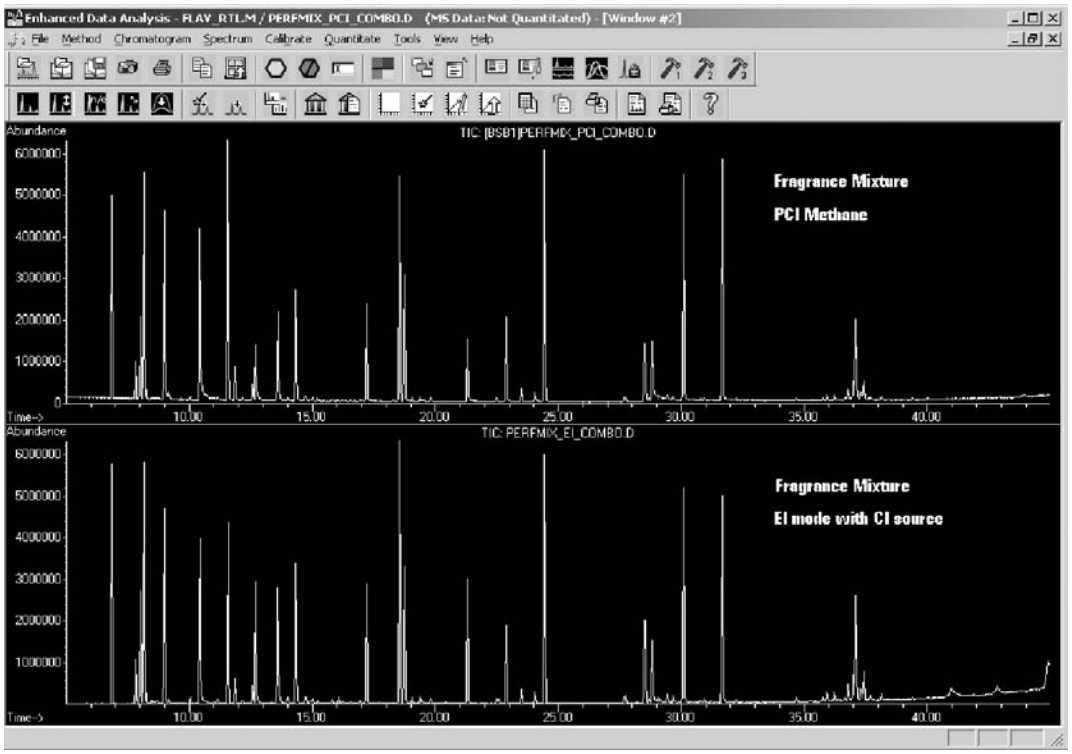

Enhanced Data Analysis - BLAV_RTLM / PERFMIX_PCI_COMBO.D (MS Data: Not Quantitated) - [Window #2]
File Method Chromatogram Spectrum Calibrate Quantitate Tools View Help
Abundance
TIC: [BSBT]PERFMIX_PCI_COMBO.D
Fragrance Mixture
PCI Methane
Time->
TIC: PERFMIX_EI_COMBO.D
Fragrance Mixture
EI mode with CI source

**Figure 1. PCI and EI total ion chromatograms using the CI source.**

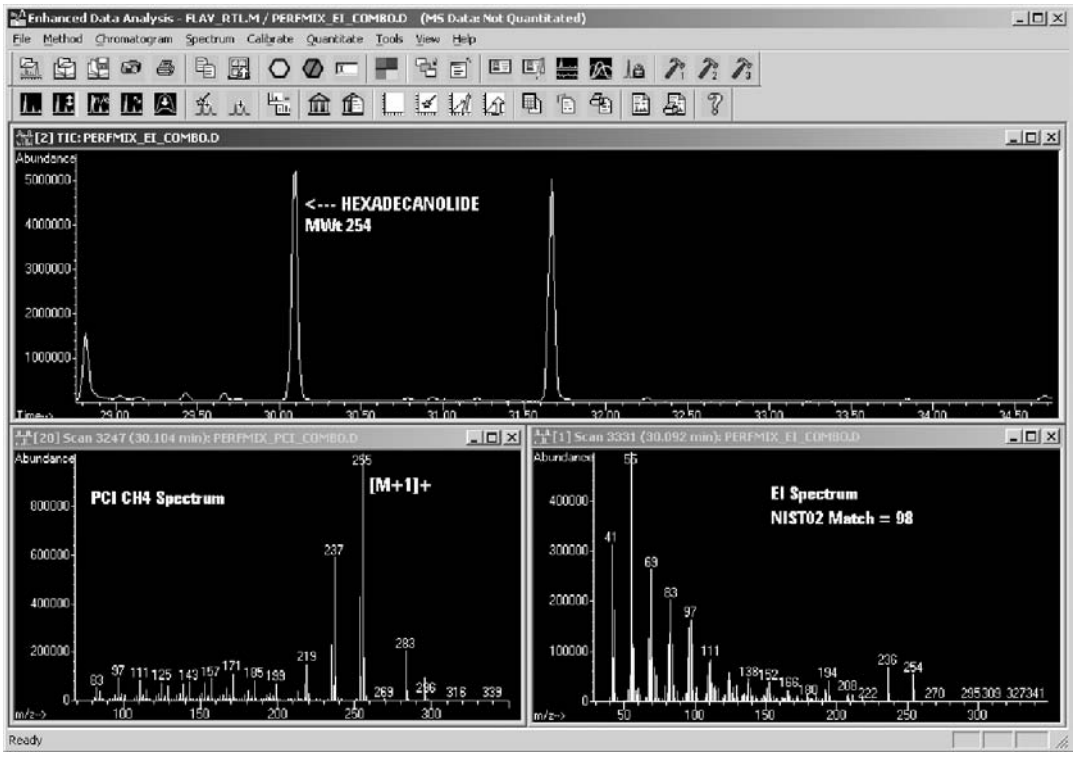

Enhanced Data Analysis - BLAV_RTLM / PERFMIX_EI_COMBO.D (MS Data: Not Quantitated)
File Method Chromatogram Spectrum Calibrate Quantitate Tools View Help
[2] TIC: PERFMIX_EI_COMBO.D
Abundance
<--- HEXADECANOLIDE
MWt 254
Time->
[20] Scan 3247 (30.104 min): PERFMIX_PCI_COMBO.D
PCI CH4 Spectrum
[M+1]+
m/z->
[1] Scan 3331 (30.092 min): PERFMIX_EI_COMBO.D
EI Spectrum
NIST02 Match = 98
Ready

**Figure 2. PCI and EI spectra for Hexadeconolide.**

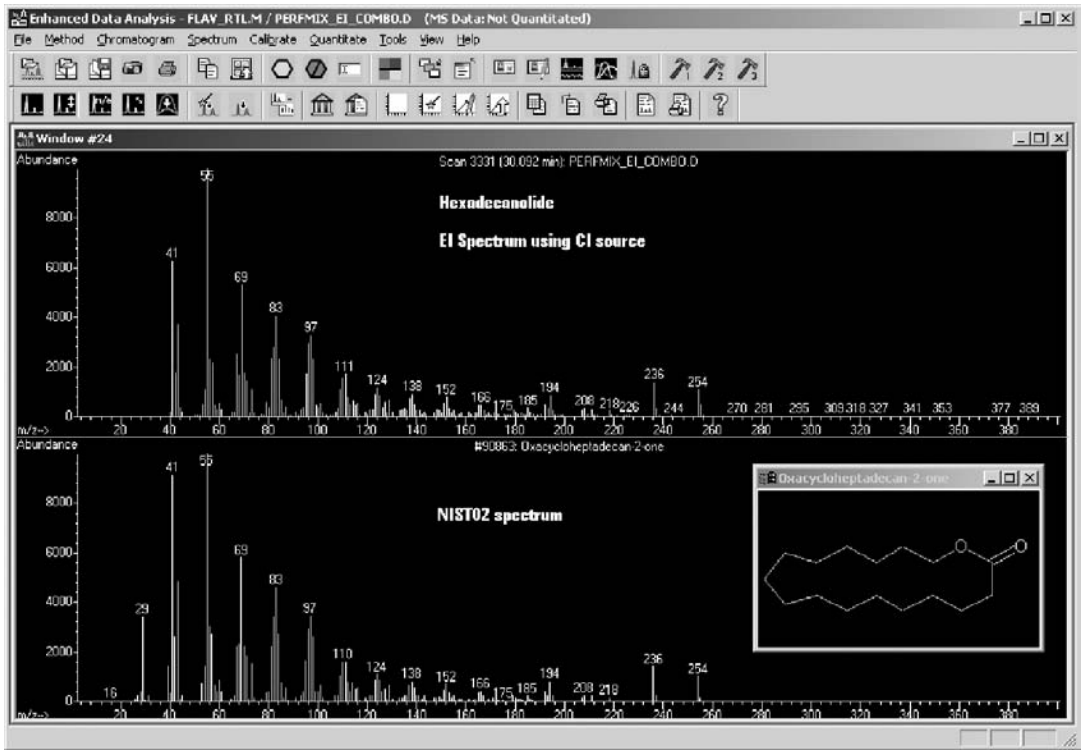



**Figure 3. Acquired EI spectrum compared to the NIST02 library reference spectrum.**

The EI data in Figure 3 shows much more fragmentation useful for compound identification. The response for 255 is relatively small. Using the NIST02 library, the EI reference spectra for Hexadecanolide (Oxacyclohelptadecan-2-one) was retrieved with a 98% quality match.

## Summary

This data demonstrates the Agilent 5973 inert GC/MSD's ability to acquire high quality EI spectra using the CI source. The EI spectra can be searched against standard libraries for identification while the CI spectra provide molecular weight information. The ability to acquire both types of data without changing sources results in increased productivity.

## For More Information

For more information on our products and services, visit our Web site at: www.agilent.com/chem

www.agilent.com/chem

The author, Chris Sandy, is a GC MS Applications Specialist for Agilent Technologies in the UK.

Agilent shall not be liable for errors contained herein or for incidental or consequential damages in connection with the furnishing, performance, or use of this material.

Information, descriptions, and specifications in this publication are subject to change without notice.

© Agilent Technologies, Inc. 2004

Printed in the USA
January 30, 2004
5989-0595EN

Agilent Technologies

# Optimizing the Agilent Technologies 6890 Series GC for High Performance MS Analysis

Technical Overview


## Author

Linda Doherty
Agilent Technologies, Inc.
Life Sciences and Chemical Analysis
5301 Stevens Creek Blvd
Santa Clara, California 95051
USA



## Abstract

**Trace level GC/MS analysis requires a system that is performing at its best. Without a properly optimized GC, the mass spectrometer may not give the sensitivity expected. In other words, when more of the sample gets from the injection port to the ion source, the more likely a detector will produce a signal. Also, if the chemical noise from the GC is too high, the signal-to-noise ratio and ability to detect small analyte concentrations will be reduced. This note is a "how-to" guide for improving the GC performance. This will, in many instances, improve the overall performance of a GC/MS system. This guide is specific for the Agilent Technologies 6890 Series GC used with the Agilent Technologies 5973 MSD.**


## Supplies

In order to improve the system performance, some supplies should be on hand. These may not give significant improvements by themselves, but when installed together, they will give the best results. Many of the referenced supplies have changed over the past few years and will continue to be improved. It is important to stay informed and purchase the most recently updated versions of the consumables. Refer to www.agilent.com/chem for the latest comsumables available.

### The Carrier Gas Line

The GC carrier gas should be at least 99.999% helium. Lower grades of helium are available and may contain impurities that can damage the GC column (for example, oxygen) and contribute to the chemical noise background. Even with a high purity gas there may be trace water, oxygen, and hydrocarbons. Putting a trap in the carrier line will eliminate these contaminants (see Figure 1). The mass spectrometer (MS) gas purifier trap is recommended and shipped with all new mass selective detectors (MSDs). It must be installed diagonally or vertically for optimum performance. Do not install it horizontally. High-purity helium also increases the effective lifetime of traps.

Precleaned, refrigeration grade 1/8-inch copper tubing should be used with high quality carrier gas. Other tubing can be cleaned by running solvents (methanol, ethyl acetate, hexane) through it in a water aspirator vacuum setup. The use of chlorinated solvents is not recommended due to

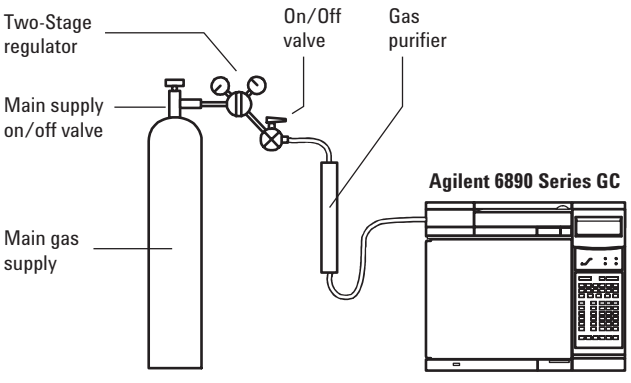



**Figure 1. General gas plumbing assembly. The MS gas purifier must be installed diagonally or vertically.**

possible long term contamination of flow lines and controllers. Another commonly used cleaning technique is to heat the copper tubing with a Bunsen burner, propane torch, or heat gun, while helium is flowing through the tubing. This is done after connecting the tubing to the helium supply but before connecting it to the GC. This process bakes off all the volatile contaminants. Take proper safety precautions while heating the tubing. Laboratory manifold systems, especially when new, tend to have hydrocarbon contaminants. Purging the new lines, before connecting the clean tubing to analytical instruments, is essential. The supplies needed for the carrier gas line are:

- ≥99.999% He: Gas supplier
- Clean copper tubing (50 ft): p/n 5180-4196
- Mass Spectrometer gas purifier: p/n RMSH-2
- Bracket to mount the gas purifier: p/n UMC-5-2

### Splitless Inlet Consumables

The capillary inlet (Figure 2) has many consumable parts that should be kept on hand. Many of these consumables, such as liners (5), come in a variety of designs (Appendix A). The proper liner to use is dependent on the application. For trace level analysis, the single tapered, deactivated liner is recommended. The Viton O-ring (4) holding the liner in place should be replaced periodically to reduce the chance of leaking. The seal and the washer in the bottom of the injection port (10, 11) should be replaced whenever the reducing nut (12) is removed. The recommended seal is gold plated to reduce metal catalyzed thermal degradation of analytes. Septa (2) should be replaced quite frequently: for example, every 100 injections. The low bleed, precored, red septa should be used. Keeping a beaker of septa in an oven at 250 °C at all times will eliminate the need to condition the septum once it is in place. A Merlin Microseal™ is highly recommended over a conventional septum nut and septum (1, 2). The Microseal eliminates the need for septa and lasts for tens of thousands of injections without leaking. It is most appropriate with the Automatic Liquid Sampler (ALS) injection tower and only works with untapered, blunt tip syringe needles. The Microseal has been improved this past year. Older seals have a maximum pressure rating of 30 psi. The new seals are rated to 100 psi. The new seal and nut are recommended. The gold nuts (stamped “303C”) are not compatible with the new seals. A gray nut, stamped “221B,” should be installed.

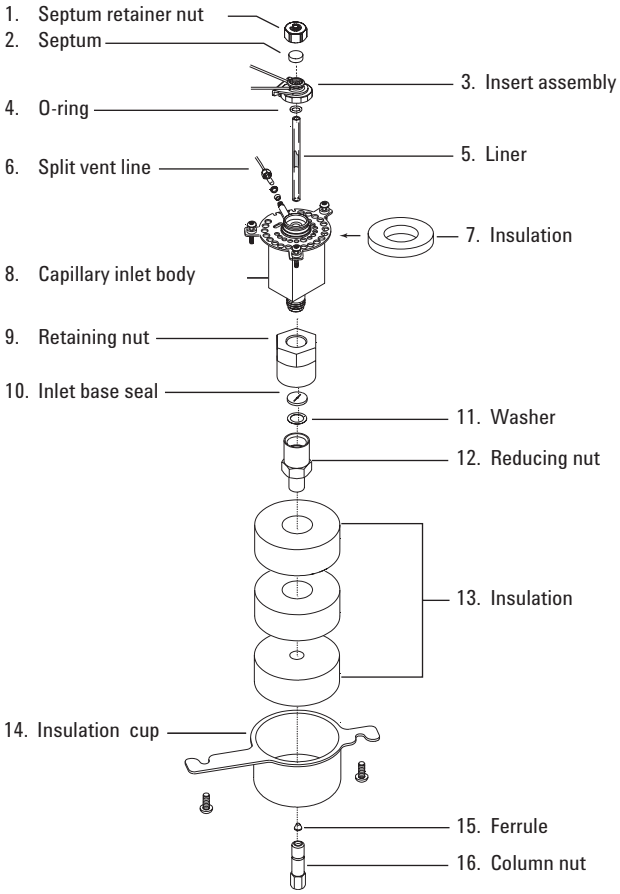



**Figure 2. Capillary inlet assembly.**

Electronic pneumatic control (EPC) is an integral part of the Agilent 6890 Series GC. The EPC version of the Agilent 6890 is required when using an Agilent 5973 MSD. The manual version of the Agilent 6890 will not work with the Agilent 5973 MSD. Electronic control gives the best repeatability in retention time and area counts. Using the electronic pulsed splitless mode allows for complete transfer of larger volume injections (up to 5 µL) onto the column. Even larger volume injections (>5 µL) may result in more inlet maintenance, especially with dirty samples.

The Agilent 6890 does have an inlet that accommodates injections up to 250 µL. It is called the programmable temperature vaporizer (PTV). It works with the ALS to deliver large volume injections (LVI). The inlet works by venting the solvent before analysis. The analyte is trapped and concentrated. It is then delivered to the column as a single plug.

The list of splitless inlet consumables is:

- Molded Septa (11 mm, red, 25/pk): p/n 5181-3383

Or

- High Pressure Merlin Microseal starter kit: p/n 5182-3442
- Merlin Microseal septum: p/n 5182-3444
- Merlin Microseal septum nut: p/n 5182-3445
- Liner, single taper, deactivated, no glass wool: p/n 5181-3316
- Viton O-ring (12/pk): p/n 5180-4182
- Gold plated seal: p/n 18740-20885
- Washer (to go with seal): p/n 5061-5869
- 10-µL Blunt needle syringe: p/n 9301-0713

#### Column Consumables

The optimal choice of column is once again dependent on the application. For trace-level, high-sensitivity applications, a column with a thin film and low bleed is best. A 30 m, 0.25-mm id, 0.25-µm film, 5% phenyl–95% methyl silicone column is a versatile column used for many applications. Special low-bleed MS columns cost more but give better results.

The proper column nut and ferrule combination are critical for a leaktight seal. Newer column nuts may not be compatible with all ferrules. The proper ferrule will be dependent on column outer diameter (od) and is specified below. The ferrule should only be slightly larger than the column od. The use of 100% graphite ferrules is not recommended, as they are easily over-tightened causing graphite to extrude into the injection port. This will be apparent when disassembling the injection port. If there are pieces of graphite in the bottom of the injection port, the ferrule(s) was/were overtightened. The presence of graphite in a hot injection port can cause thermally labile compounds to degrade. It can also affect the chromatography and cause tailing. Thus, 10% graphite, 90% Vespel ferrules are highly recommended. Vespel® ferrules will shrink as they are heated. Conditioning them for 4 hours in a 250 °C oven will preshrink them before use. Alternatively, the column nuts (Figure 2, number 16) can be retightened after the column oven cycles a few times.

The column, column nut, and ferrules supplies include:

- 30-m column, 0.25-mm id, 0.25 µm, low bleed (HP-5MS): p/n 19091S-433
- Column nuts (wrench-tighten only) 2/pk: p/n 5181-8830
- Ferrules for 0.2-mm id columns, 10/pk: p/n 5062-3516
- Ferrules for 0.25-mm id columns, 10/pk: p/n 5181-3323
- Ferrules for 0.32-mm id columns, 10/pk: p/n 5062-3514
- Ceramic scoring wafer (column cutter), 4/pk: p/n 5181-8836

A sharp column-cutting tool is needed for making clean cuts. The ceramic scoring wafers or sapphire square edge pens are desirable. The diamond point pens are harder to use. Ceramic scoring wafers are extremely sharp. They should be used with care. An X-ACTO® or Swiss Army knife is not a column cutting tool.

Use a 10× magnifier to assure that the cut is clean and no column shards are lodged inside the column.

#### Interfacing the Column to the MS

The column is connected to the MS through an interface that is sealed with a column nut and ferrule. The specific ferrule used depends on the column diameter. Never use a 100% graphite ferrule. Similar to the injection port, pieces of graphite may extrude into the interface and contaminate the MS. The ferrules required are 15% graphite, 85% Vespel. The column nut listed is brass; stainless steel nuts should never be substituted. Stainless nuts may damage the threads on the interface. Damaged threads cause air leaks and replacement of the entire interface.

The MS interface supplies include:

- Brass column nut: p/n 05988-20066
- Ferrules for 0.2 and 0.25-mm id columns, 10/pk: p/n 5062-3508
- Ferrules for 0.32-mm id columns, 10/pk: p/n 5062-3506

## Installation of Consumables

This section assumes that you are going to perform preventative maintenance (PM) on your Agilent 6890 Series GC. If this is a new gas chromatography/mass selective detector (GC/MSD) system, many of these steps will be completed by a Customer Engineer during installation. Before beginning the PM, please read this section carefully. The necessary manuals will be referenced frequently. These manuals can be downloaded from our Web site at www.agilent.com/chem.

The manuals necessary include:

- Agilent 6890N GC User Information Manual: G1530-90210
- Agilent 6890 Series GC Service Manual: G1530-90220
- Agilent 5973 Series MSD Hardware Installation Manual: G2589-90006
- Agilent 5973 inert MSD Hardware Manual: G2589-90071
- Agilent 5973 Series MSD Site Preparation Guide: G2589-90070

With all of the consumable supplies previously mentioned at hand, a proper PM can be completed. To begin a PM, it is necessary to cool the GC zones (oven, inlet, MS interface). Vent the Agilent 6890 Series MSD. Please refer to the MS hardware manuals for venting instructions.

#### Installation of Gas Supplies

Follow the directions in the GC site preparation/installation manual and install the gas line supplies. Take care in making the Swagelok connections. The trap can break if too much stress is placed on the connection during tightening. Leak check all connections with a helium leak detector. (No Snoop please!) Make sure to purge all lines with helium before connecting them to the GC.

#### Installation of Capillary Inlet Supplies

Before handling any of the injection port supplies, wash hands and/or wear lint-free gloves. Oils on the hands will be transferred to these parts and become background in the system, requiring extra bakeout time. Following the instructions in the GC operating manual, remove the septum nut, septum, and liner. Discard the septum, liner, and liner O-ring. Open the oven door, loosen the 1/16-inch column nut, and remove the column and nut.

Remove the insulation cup and any necessary insulation (Figure 2, number 14) to provide access to the reducing nut (Figure 2, number 12). If the lower insulation cup was not in place, find it, because this piece improves the inlet temperature profile.

With a 1/2-inch wrench, remove the reducing nut (Figure 2, number 12). Due to heat cycling of the GC, the reducing nut will be very tight. Remove the seal and the washer (Figure 2, number 10, 11) and discard. Place a new washer in the reducing nut and a new seal (flat side with groove up). Hand-tighten the reducing nut back into place within the retaining nut and then wrench-tighten until very tight. Replace the insulation cup. Insert a new liner and O-ring. The single taper liners are installed with the taper down, toward the column end of the inlet. Hand-tighten the insert assembly (Figure 2, number 3). Add the Merlin Microseal or proper preconditioned septum and septum nut. The molded septum is installed with the hole up. Follow the directions supplied with the Merlin Microseal to insure proper installation. If the green septum nut is used, wrench-tighten the weldment and septum nut with the septum nut wrench until the C-ring lifts off the top of the green septum nut.

At this point, the inlet should be leak checked. Follow the directions in the GC maintenance and troubleshooting section of the manual.

#### Column Installation

Working with fused silica columns may be dangerous. Wear proper eye protection. Inspect the column for damage or breakage. Unweave 0.5–1 coil of the column from its basket to make it easier to install. Push a septum onto the inlet end of the column about 10 cm. Put the column nut and appropriate ferrule on the column. Cut 5–10 cm off the inlet end of the column. Check the cut with a 10× magnifier; the cut should be straight, not jagged, with no column shards within the column. If the cut is jagged or shards are inside, try again. After a clean cut is obtained, mark the proper column position with the septum (Figure 3). The septum will hold the column nut and ferrule in place. Place the column on the column hanger. Insert the column nut into the inlet reducing nut and finger-tighten. Wrench-tighten the column nut. The column should be stationary in the ferrule. Carefully slide the septum down away from the nut without disturbing the column positioning. The septum can be left in place if desired. Using the GC keypad or the MS ChemStation software, input the

**Table 1. Head Pressures and Calculated Flowrates for a Splitless Inlet at an Oven Temperature of 25 °C with the Outlet Pressure set to Vacuum**

| Column id (mm) | Length (meters) | Head pressure (psi) | Linear velocity (cm/s) | Column flow (mL/min) |
|---|---|---|---|---|
| 0.20 | 12 | 6.0 | 57 | 1.0 |
| 0.20 | 25 | 15.0 | 39 | 1.0 |
| 0.20 | 50 | 28.0 | 28 | 1.0 |
| 0.25 | 30 | 6.2 | 36 | 1.0 |
| 0.32 | 30 | 3.4 | 50 | 2.0 |
| 0.32 | 50 | 5.5 | 34 | 1.5 |

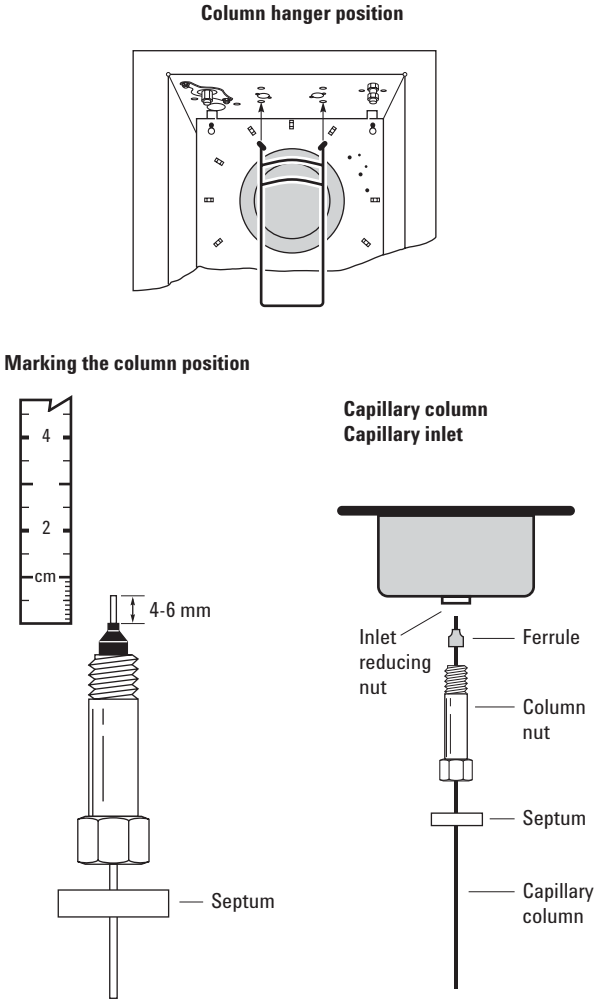



**Figure 3. Proper installation of capillary columns in a capillary inlet.**

Column Dimensions, set Outlet Pressure to Vacuum and Column Flow between 1 and 1.5 mL/min helium (Table 1). The Split Vent Flow should be approximately 50 mL/min. These parameters are accessed through the inlet and column screens. Place the detector end of the column into a beaker of water and check for bubbles to show helium flow. Heat the injection port. When the injection port reaches the setpoint temperature, retighten the column nut in case it loosened. Check once again for column flow. Remove the end of the column from the beaker and close the oven door. Condition the column by slowly (5 °C/min) ramping it to its maximum operating temperature. Leave it at that temperature for least 2 hours; overnight is preferable. The maximum operating temperature for an HP-5MS column is 325 °C. Cool the oven to ambient and insert the interface nut and ferrule onto the column. Properly cut off 5–10 cm of the column. Properly place the column into the interface by following the directions in the MS hardware manual for the Agilent 5973 and for the Agilent 6890 series MSD. Hand-tighten the interface nut and then wrench-tighten the nut. The nut should be tightened only until you hear two squeaks. This is a firm seal. Pump down the detector as directed by the MSD manual (for the Agilent 5973 and for the Agilent 6890 Series MSD). Keep the oven at ambient temperature until the source is hot. Check the interface connection after the interface is heated. The interface nut may need additional tightening.

## Tips for Better Method Performance

Numerous splitless parameters need to be optimized for the best splitless injection. Those parameters include:

- Injection port temperature
- Column flow
- Liner design
- Solvent
- Sample volume
- Sample volatility
- Splitless valve time
- Injection speed

The proper inlet temperature is needed to evaporate high boiling point compounds without thermally degrading other compounds. Normally, the inlet temperature is a compromise between these two factors. A good starting point is 250 °C.

Liner design is one of the most difficult choices simply because of the variety of liners available. The features that are most important in a liner are the volume, whether it is deactivated or not, and whether or not it contains deactivated glass wool. As a general choice for high sensitivity work, a 4-mm single tapered, deactivated liner with no glass wool is recommended. For large volume injections (≥2 µL) and for the highest repeatability (especially with small volume injections of ≤0.5 µL [1]), deactivated glass wool is necessary. For dirty samples, deactivated glass wool helps to keep the nonvolatiles from getting to the column, but too much deactivated glass wool can greatly decrease sensitivity. Often, the most appropriate liner must be determined through experimentation. Please note: Removing and/or breaking deactivated glass wool creates active sites.

Splitless valve timing is critical. The ON time (splitless mode) should to be long enough to assure that all of the injected sample reaches the column.

A textbook splitless injection has the liner volume swept at least two times (during the "ON" time). A 4-mm liner has an approximate volume of 1 mL. With a GC/MS flow rate of 1 mL/min, a 2-min splitless injection would be necessary.

However, this long splitless time has not been common. There are two reasons for this:

- Conventional (manually controlled) capillary inlets were pressure regulated (constant pressure, regardless of oven temperature) and not flow regulated (changing pressure with oven temperature), so a higher-than-optimal flow was set initially so that the flow did not go to zero at high oven temperatures. Thus, a typical splitless or "OFF" time has been between 0.5 to 1.5 min.
- Liner volumes smaller than the textbook examples have typically been used. Since a 2-mm liner (250 µL volume) was more commonly employed, the splitless time was proportionally shorter.

Finally, with the programmable control afforded by EPC, flows can be reliably pulsed during the injection process. With pulsed splitless injections, flows during the splitless time can be 2-6 mL/min, resulting in splitless times less than 2 min for a 4-mm id liner. The pulsed splitless injection mode on the Agilent 6890 is recommended for GC/MS work. After the injection pulse, the system returns to analytical flow rates of 1–4 mL/min He. The highest flow allowable depends on the MSD. Refer to the appropriate MS hardware manual for your detector's limit.

Unless all analytes have high boiling points, the initial oven temperature should be set to take advantage of the solvent effect. The solvent effect focuses the analytes on the head of the column. The oven temperature should typically be set to ≥10 °C below the boiling point of the solvent used (Table 2). On the other hand, the MS interface temperature should be hot enough to avoid loss of analytes on cold spots. The interface should be set to the maximum oven temperature for the analysis or 10 °C–15 °C higher if the upper temperature limit for the column is not exceeded. The default method temperature is 280 °C; the interface temperature should be optimized as part of method development.

Finally, the rate of auto-injection of a sample has been studied for splitless injections. It has been found that fast injections, such as with the ALS, tend to give the most repeatable and non-discriminating results.

**Table 2. Boling and Initial Oven Temperatures for Common Solvents**

| Solvent | Boiling point (°C) | Initial oven temperature (°C) |
|---|---|---|
| Diethyl ether | 36 | 10 to 25 |
| n-Pentane | 36 | 10 to 25 |
| Methylene chloride | 40 | 10 to 35 |
| Carbon disulfide | 46 | 10 to 35 |
| Acetone | 56 | 25 to 45 |
| Chloroform | 61 | 25 to 50 |
| Methanol | 65 | 35 to 55 |
| n-Hexane | 69 | 40 to 60 |
| Ethyl acetate | 77 | 45 to 65 |
| Acetonitrile | 82 | 50 to 70 |
| n-Heptane | 98 | 70 to 90 |
| Isooctane | 99 | 70 to 90 |
| Toluene | 111 | 80 to 100 |

## Using Pulsed Splitless Injections

Pulsed splitless injections are the best way to do splitless injections. EPC of the splitless inlet allows for high flow rates initially, followed by more typical GC/MS flow rates. A pulsed splitless injection transfers more of the sample onto the column and allows for increased injection volumes up to 5 µL. When the injected volume is flash vaporized, the required expansion volume for the solvent is greatly increased (solvent choice also effects expansion volume). The use of higher initial inlet pressures reduces the volume ($P_1V_1 = P_2V_2$) so the entire injected volume can move to the column. The higher pressure also decreases the likelihood that highly volatile compounds will escape out the top of the injection port through the septum purge vent (Figures 4 and 5). In the case of thermally labile compounds, the faster they leave the hot injection port the less likely they are to degrade [2, 3]. The flow rate is then reduced to the value desired for the chromatographic separation. This flow is held constant by increasing the pressure as the oven temperature increases. Figure 6 is a graphical representation of the pulsed splitless technique with constant flow. Pulsed splitless injections should always be used when sensitivity and/or repeatability are critical. Refer to the GC operating manual for how to set up a pulsed splitless injection.

Electronic pressure and flow control of carrier gases not only help with larger volumes, they also help to decrease run times and maintain stable MS sensitivity by keeping the carrier gas flow constant. These lead to shorter analyses, higher sensitivity, and higher reproducibility. The benefits for the analyst are more chromatographic analyses per shift, better data, and higher revenues per instrument.

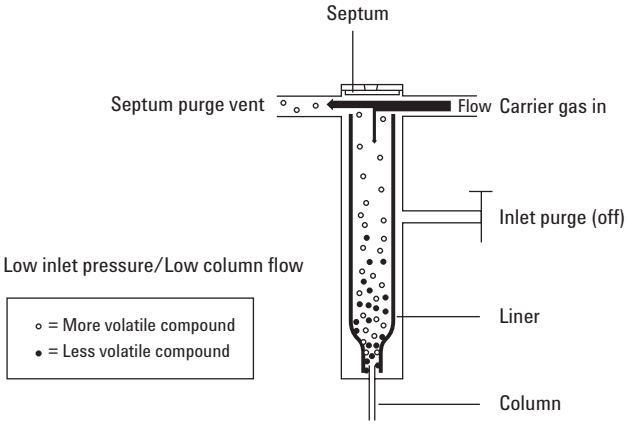



**Figure 4. A low initial inlet pressure causes loss of volatile compounds.**

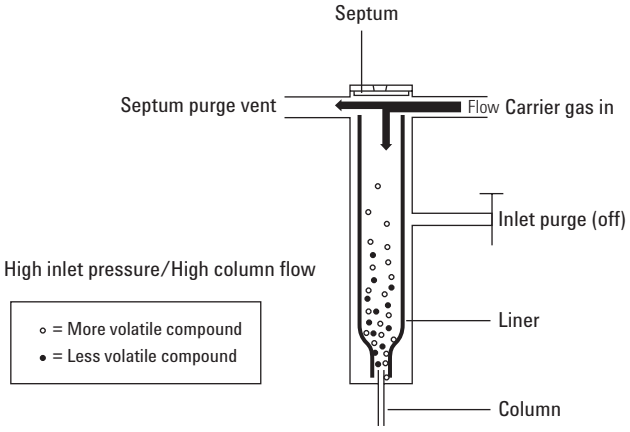



**Figure 5. With the correct inlet pressure there is no loss of volatile compounds.**

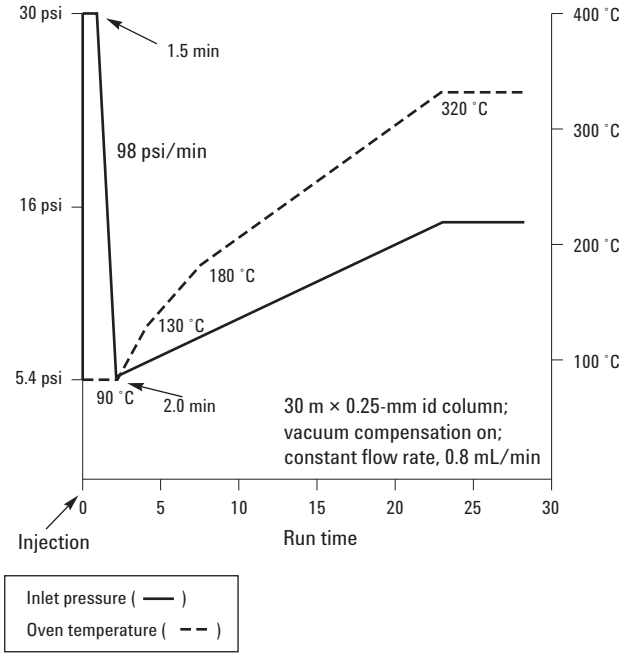



**Figure 6. Pulsed splitless injection technique employing constant flow with EPC. This technique allows larger injection volumes and inhibits the loss of volatile compounds out of the septum purge vent.**

## Summary

Following the instructions in this guide will improve analytical results with your GC/MSD system. Contamination interferring with the determination of your analytes will be minimized and sample transfer optimized, both improving sensitivity.

A final note: the choice of vials and septa will affect your results. Screw cap vials are not recommended for GC/MS analyses. Application note "Effects of Vial Septa Used in GC/ECD Analysis of Trace Organics" Agilent Technologies publication 5091-8980E www.agilent.com/chem will help you make the right choice.

## References

1. "Analyzing aromatics in reformulated gasoline by GC/MS", Agilent Technologies, publication (23) 5964-0116E.
2. Philip L. Wylie et al. Improving the Analysis of Pesticides by Optimizing Splitless Injections. 1995 Pittsburgh Conference paper number 347.
3. Philip L. Wylie and Katsura Uchiyama. Improved Gas Chromatogaphic Analysis of Organophoshorous Pesticides with Pulsed Splitless Injection. (1996) *JAOAC* **79**: 571-577.

# Appendix A

### Capillary Inlet System Liners

| Liner | Part no. | Price | Configuration: ID volume | Glass type | Deactivated | Glass wool packing** | Applications: Fast injection (7673 ALS) Split | Fast injection (7673 ALS) Split-less | Slow & manual injection Split | Slow & manual injection Split-less | Linear seals: Fluoro-carbon (Max 350 °C) | Linear seals: Graphite (350 °C and above) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Single-Taper liner | 5062-3587 | 34 | 4 mm (0.8-mm end) 900 L | Borosilicate | YES | YES** | C | ● | C | ● | 5180-4182 (12/pk) 12 | 5180-4173 (12/pk) 48 |
| Single-Taper liner | 5181-3316 | 31 | 4 mm (0.8-mm end) 900 L | Borosilicate | YES | NO | — | A | — | ● | 5180-4182 (12/pk) 12 | 5180-4173 (12/pk) 48 |
| Double-Taper liner | 5181-3315 | 34 | 4 mm (0.8-mm end) 800 L | Borosilicate | YES | NO | — | A | — | ● | 5180-4182 (12/pk) 12 | 5180-4173 (12/pk) 48 |
| Capillary liner | 19251-60540 | 26 | 4 mm 990 L | Borosilicate | NO | YES** | ● | D | ● | D | 5180-4182 (12/pk) 12 | 5180-4168 (12/pk) 48 |
| Split liner | 18740-60840 | 46 | 4 mm with cup | Borosilicate | NO | YES + column packing | — | — | E | — | 5180-4182 (12/pk) 12 | 5180-4168 (12/pk) 48 |
| Split liner | 18740-80190 | 41 | 4 mm with cup | Borosilicate | NO | NO | — | — | ● | — | 5180-4182 (12/pk) 12 | 5180-4168 (12/pk) 48 |
| Splitless liner | 18740-80220 | 27 | 2 mm 250 L | Quartz (Purity 8) | NO | NO | — | A,B | — | B | 5180-4182 (12/pk) 12 | 5180-4173 (12/pk) 48 |
| Splitless liner | 5181-8818 | 30 | 2 mm 250 L | Quartz (Purity 8) | YES | NO | — | A,B | — | B | 5180-4182 (12/pk) 12 | 5180-4173 (12/pk) 48 |
| Direct liner | 18740-80200 | 17 | 1.5 mm 140 L | Borosilicate | NO | NO | — | A,B | — | B | 5180-4182 (12/pk) 12 | 5180-4173 (12/pk) 48 |

● General recommendation

○ See notes at left regarding use

* Quality discounts available; please inquire.

** Silanized glass wool, 10 gm, (pesticide grade) (Agilent p/n 5181-3317).

A. Can be used without the glass wool in some applications, but liners with glass wool are generally recommended for best reproducibility in fast injections.

B. Recommended only for small (< 0.5 µL) volumes, depending on solvent and conditions.

C. The glass wool packing as supplied may not provide adequate mixing for good precision in split injections. Liners can be packed with silanized glass wool positioned as in the straight 4-mm capillary liner, part number 19251-60540.

D. Taper liners are recommended for best performance in this application, particularly with labile samples and wide-boilingrange mixtures.

E. Not recommended for use with EPC.

## For More Information

For more information on our products and services, visit our Web site at: www.agilent.com/chem

Agilent shall not be liable for errors contained herein or for incidental or consequential damages in connection with the furnishing, performance, or use of this material.

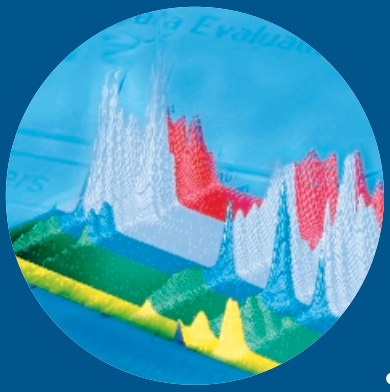
Information, descriptions, and specifications in this publication are subject to change without notice.

Microseal™ is a trademark of the Merlin Instrument Company.
Swagelok™ is a registered trademark of the Swagelok Complany.
Snoop™ is a registered trademark of the Nupro Company
Vespel® is a registered trademark of E. I. du Pont de Nemours Co. (Inc.)
X-Acto® is a registered trademark of the Hunt Corporation.

© Agilent Technologies, Inc. 2003

Printed in the USA
November 14, 2003
5988-9944EN

Agilent Technologies



# HPLC for Food Analysis

## A Primer

Agilent Technologies

© Copyright Agilent Technologies Company, 1996-2001.
All rights reserved. Reproduction, adaption, or translation without prior written permission is prohibited, except as allowed under the copyright laws.

Printed in Germany
September 01, 2001

Publication Number 5988-3294EN

www.agilent.com/chem

Agilent Technologies

# HPLC for Food Analysis

## A Primer

The fundamentals of an alternative approach to solving tomorrow's measurement challenges

Angelika Gratzfeld-Hüsgen and Rainer Schuster

**Acknowledgements**

We would like to thank Christine Miller and John Jaskowiak for their contributions to this primer. Mrs. Miller is an application chemist with Agilent Technologies and is responsible for the material contained in chapter 5. Mr. Jaskowiak, who wrote chapter 7, is a product manager for liquid chromatography products at Agilent Technologies.

© Copyright Agilent Technologies Company 1996-2001. All rights reserved. Reproduction, adaption, or translation without prior written permission is prohibited, except as allowed under the copyright laws.
Printed in Germany, September 1, 2001.
Publication Number 5988-3294EN

# Preface

Modern agriculture and food processing often involve the use of chemicals. Some of these chemicals and their functions are listed below:

- Fertilizers: increase production of agricultural plants
- Pesticides: protect crops against weeds and pests
- Antibiotics: prevent bacteria growth in animals during breeding
- Hormones: accelerate animal growth
- Colorants: increase acceptability and appeal of food
- Preservatives and antioxidants: extend product life
- Natural and artificial sweeteners and flavors: improve the taste of food
- Natural and synthetic vitamins: increase the nutritive value of food
- Carbohydrates: act as food binders

Such chemicals improve productivity and thus increase competitiveness and profit margins. However, if the amounts consumed exceed certain limits, some of these chemicals may prove harmful to humans.

Most countries therefore have established official tolerance levels for chemical additives, residues and contaminants in food products. These regulations must be monitored carefully to ensure that the additives do not exceed the prescribed levels. To ensure compliance with these regulatory requirements, analytical methods have been developed to determine the nature and concentration of chemicals in food products. Monitoring of foodstuffs includes a check of both the raw materials and the end product. To protect consumers, public control agencies also analyze selected food samples.

High-performance liquid chromatography (HPLC) is used increasingly in the analysis of food samples to separate and detect additives and contaminants. This method breaks down complex mixtures into individual compounds, which in turn are identified and quantified by suitable detectors

and data handling systems. Because separation and detection occur at or slightly above ambient temperature, this method is ideally suited for compounds of limited thermal stability. The ability to inject large sample amounts (up to 1–2 ml per injection) makes HPLC a very sensitive analysis technique. HPLC and the nondestructive detection techniques also enable the collection of fractions for further analysis. In addition, modern sample preparation techniques such as solid-phase extraction and supercritical fluid extraction (SFE) permit high-sensitivity HPLC analysis in the ppt (parts per trillion) range. The different detection techniques enable not only highly sensitive but also highly selective analysis of compounds.

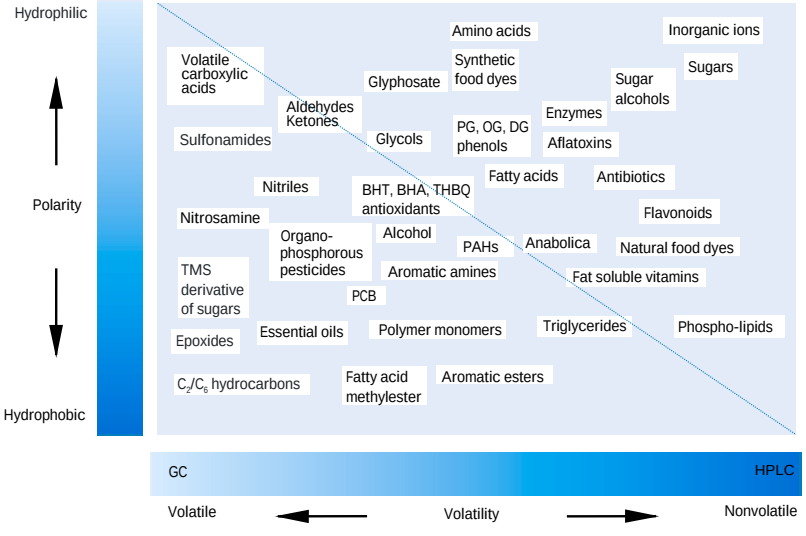



**Figure 1**
**Match of analyte characteristics to carrier medium**

Its selective detectors, together with its ability to connect a mass spectrometer (MS) for peak identification, make gas chromatography (GC) the most popular chromatographic method.

HPLC separates and detects at ambient temperatures. For this reason, agencies such as the U.S. Food and Drug Administration (FDA) have adopted and recommended HPLC for the analysis of thermally labile, nonvolatile, highly polar compounds.

Capillary electrophoresis (CE) is a relatively new but rapidly growing separation technique. It is not yet used in the routine analysis of food, however. Originally CE was applied primarily in the analysis of biological macromolecules, but it also has been used to separate amino acids, chiral drugs, vitamins, pesticides, inorganic ions, organic acids, dyes, and surfactants.[1, 2, 3]

Part 1 is a catalog of analyses of compounds in foods. Each section features individual chromatograms and suggests appropriate HPLC equipment. In addition, we list chromatographic parameters as well as the performance characteristics that you can expect using the methods shown. In part 2 we examine sample preparation and explain the principles behind the operation of each part of an HPLC system—sampling systems, pumps, and detectors—as well as instrument control and data evaluation stations. In the last of 11 chapters, we discuss the performance criteria for HPLC, which are critical for obtaining reliable and accurate results. Part 3 contains a bibliography and an index.

# Contents

## Part One
## The HPLC Approach


**Chapter 1 Analytical examples of food additives**

Acidulants . . . . . 2
Antioxidants . . . . . 4
Preservatives . . . . . 6
Artificial sweeteners . . . . . 8
Colorants . . . . . 10
Flavors . . . . . 12
Vanillin . . . . . 12
Bitter compounds: hesperidin and naringenin . . . . . 14

**Chapter 2 Analytical examples of residues and contaminants**

Residues of chemotherapeutics and antiparasitic drugs . . 16
Tetracyclines . . . . . 18
Fumonisins . . . . . 19
Mycotoxins . . . . . 21
Bisphenol A diglydidyl-ether (BADGE) . . . . . 24
Pesticides . . . . . 26
Carbamates . . . . . 28
Glyphosate . . . . . 29

**Chapter 3 Analytical examples of natural components**

Inorganic anions . . . . . 32
Lipids . . . . . 35
Triglycerides and hydroperoxides in oils . . . . . 35
Triglycerides in olive oil . . . . . 37
Fatty acids . . . . . 38
Carbohydrates . . . . . 40
Vitamins . . . . . 42
Water-soluble vitamins . . . . . 42
Fat-soluble vitamins . . . . . 45
Analysis of tocopherols on normal-phase column . . . . 46
Biogenic amines . . . . . 48
Amino acids . . . . . 50
Peptides . . . . . 52

## Part Two
## The Equipment Basics


**Chapter 4 Separation in the liquid phase**

Separation mechanisms . . . . . 58
- Reversed-phase materials . . . . . 58
- Ion-exchange materials . . . . . 58
- Size-exclusion gels . . . . . 59
- Adsorption media . . . . . 59

The advent of narrow-bore columns . . . . . 59
- Influence of column temperature on separation . . . . . 60

**Chapter 5 Sample preparation**

Sample preparation steps . . . . . 62
- Automation . . . . . 62

Solids . . . . . 63
- Ultrasonic bath liquid extraction . . . . . 63
- Steam distillation . . . . . 64
- Supercritical fluid extraction . . . . . 64

Liquids . . . . . 65
- Liquid-liquid extraction . . . . . 65
- Solid-phase extraction . . . . . 65
- Gel permeation chromatography . . . . . 66
- Guard columns . . . . . 67

**Chapter 6 Injection techniques**

Characteristics of a good sample introduction device . . . 70

Manual injectors . . . . . 71

Automated injectors . . . . . 72

Autosampler with sample pretreatment capabilities . . . . 72
- Derivatization . . . . . 73

**Chapter 7 Mobile phase pumps and degassers**

Characteristics of a modern HPLC pump . . . . . 76
- Flow ranges . . . . . 76
- Gradient elution . . . . . 76
- Gradient formation at high pressure . . . . . 77
- Gradient formation at low pressure . . . . . 77

Pump designs for gradient operation . . . . . 78
Low-pressure gradient Agilent 1100 Series pump . . . . 78
High-pressure gradient Agilent 1100 Series pump . . . . 80
Degassing . . . . . 82
Helium degassing . . . . . 83
Vacuum degassing . . . . . 84

**Chapter 8 Detectors**

Analytical parameters . . . . . 87
Limit of detection and limit of quantification . . . . . 87
Selectivity . . . . . 87
Linearity . . . . . 88
Qualitative information . . . . . 88
UV detectors . . . . . 89
Diode array detectors . . . . . 90
Three dimensions of data . . . . . 91
Fluorescence detectors . . . . . 95
Cut-off filter . . . . . 96
Signal/spectral mode . . . . . 96
Online spectral measurements and
multi signal acquisition . . . . . 96
Multisignal . . . . . 97
Electrochemical detectors . . . . . 98
Electrode materials . . . . . 99
Flow cell aspects . . . . . 99
Automation features . . . . . 100
Mass spectrometers . . . . . 101
API interfaces . . . . . 102
Refractive index detectors . . . . . 104

**Chapter 9 Derivatization chemistries**
Addition of UV-visible chromophores . . . . . 108
Addition of a fluorescent tag . . . . . 109
Precolumn or postcolumn? . . . . . 109
Automatic derivatization . . . . . 110

**Chapter 10 Data collection and evaluation techniques**
Strip chart recorders . . . . . 112
Integrators . . . . . 113
Personal computers . . . . . 114
Local area networks . . . . . 117
Networked data systems . . . . . 118

**Chapter 11 Factors that determine performance in HPLC**
Limit of detection and limit of quantification . . . . . 121
Accuracy and precision . . . . . 122
Qualitative information . . . . . 123

**Part Three References and Index**

**References** . . . . . 125

**Index** . . . . . 129

# The HPLC Approach

# Part One

A demonstration of liquid chromatographic separations in food analysis

*Chapter 1*

# Analytical examples of food additives

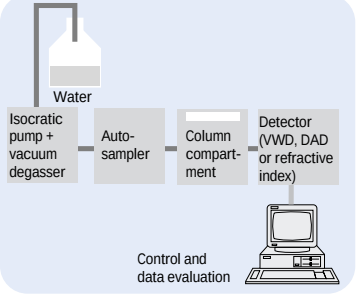
## Acidulants

Sorbic acid and citric acids are commonly used as acidulants[4] and/or as preservatives. Acetic, propionic, succinic, adipic, lactic, fumaric, malic, tartaric, and phosphoric acids can serve as acidulants as well. Acidulants are used for various purposes in modern food processing. For example, citric acid adds a fresh, acidic flavor, whereas succinic acid gives food a more salty, bitter taste. In addition to rendering foods more palatable and stimulating, acidulants act as

- flavoring agents to intensify certain tastes and mask undesirable aftertastes
- buffering agents to control the pH during food processing and of the finished products
- preservatives to prevent growth of microorganisms
- synergists to antioxidants to prevent rancidity and browning
- viscosity modifiers in baked goods
- melting modifiers in cheese spreads and hard candy
- meat curing agents to enhance color and flavor

### Sample preparation

Sample preparation depends strongly on the matrix to be analyzed, but in general steam distillation and solid-phase extraction techniques can be used.




### Chromatographic conditions

High-performance liquid chromatography (HPLC) with UV-visible diode-array detection (UV-DAD) has been applied in the analysis of citric acid in wine and in a vodka mixed drink. Retention time and spectral data were used as identification tools.

| | |
|---|---|
| Sample preparation | filtration |
| Column | 300 x 7.8 mm BioRad HPX 87-H, 9 µm |
| Mobile phase isocratic | 0.0035 M $H_2SO_4$ |
| Flow rate | 0.6 ml/min |
| Column compartment | 65 °C |
| Injection volume | 10 µl |
| Detector | UV-VWD detection wavelength 192 nm or 210 nm |

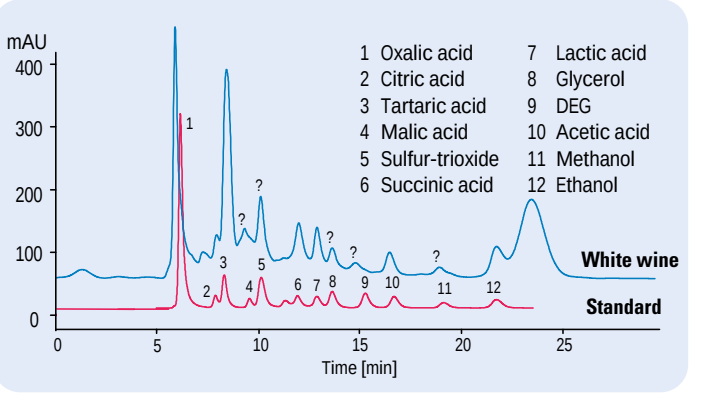



**Figure 2**
**Analysis of acidulants in white wine**

| | |
|---|---|
| Conditions as above except | |
| Mobile phase isocratic | 0.007 M $H_2SO_4$ |
| Detector | UV-DAD |

**HPLC method performance**

| | |
|---|---|
| Limit of detection | 100 ng injected amount, S/N = 2 equivalent to 2 ppm with 50 µl injected volume |
| Repeatability of | |
| RT over 10 runs | < 0.1 % |
| areas over 10 runs | < 3 % |

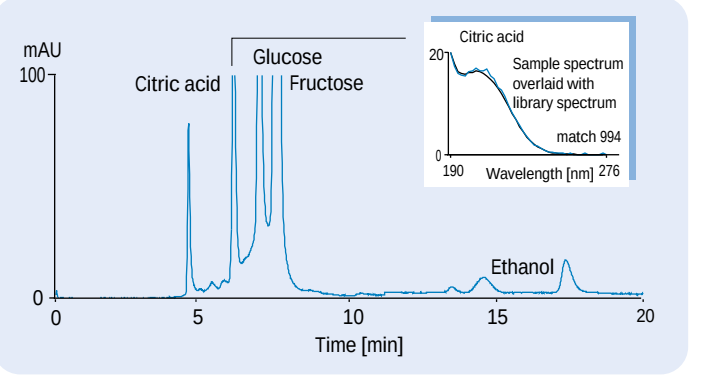



**Figure 3**
**Analysis of citric acid in vodka**

*4. Official Methods of Analysis, Food Compositions; Additives, Natural Contaminants,* 15th ed; AOAC: Arlington, VA, **1990**, Vol. 2.; Official Method AOAC 986.13: quinic, malic, citric acid in cranberry juice cocktail and apple juice.

# Antioxidants

The following compounds are used as antioxidants in food products:[4]

Natural antioxidants:

- vitamin C
- vitamin E

Synthetic antioxidants:

| | |
|---|---|
| • BHT | butylated hydroxytoluene |
| • BHA | butylated hydroxyanisole |
| • TBHQ | mono-tert-butylhydroquinone |
| • THBP | 2,4,5-trihydroxybutyrophenone |
| • PG | propyl gallate |
| • OG | octyl gallate |
| • DG | dodecyl gallate |
| • Ionox-100 | 4-hydroxymethyl-2,6-di(tert-butyl)phenol |
| • NDGA | nordihydroguaiaretic acid |
| • TDPA | 3,3'-thiodipropionic acid |
| • ACP | ascorbyl-palmitate |

Antioxidants may be naturally present in food, or they may be formed by processes such as smoking. Examples of natural antioxidants include tocopherols (vitamin E) and acsorbic acid (vitamin C). A second category of antioxidants comprises the wholly synthetic antioxidants. When these antioxidants are added to foodstuffs, they retard the onset of rancidity by preventing the oxidative degradation of lipids. In most countries where antioxidants are permitted either singly or as combinations in foodstuffs, maximum levels for these compounds have been set.

### Sample preparation

Sample preparation depends strongly on the matrix to be analyzed. For samples low in fat, liquid extraction with ultrasonic bath stimulation can be used. For samples with more complex matrices, solid-phase extraction, liquid/liquid extraction, or steam distillation may be necessary.

## Chromatographic conditions

HPLC and UV-visible diode-array detection have been applied in the analysis of antioxidants in chewing gum. Spectral information and retention times were used for identification.

| | |
|---|---|
| Sample preparation | ultrasonic liquid extraction with acetonitrile (ACN) |
| Column 1 | 100 x 4 mm BDS, 3 µm |
| Mobile phase | A = water + 0.2 ml $H_2SO_4$, pH = 2.54<br>B = ACN |
| Gradient | start with 10 % B<br>at 3 min 60 % B<br>at 4 min 80 % B<br>at 11 min 90 % B |
| Flow rate | 0.5 ml/min |
| Post time | 4 min |
| Column compartment | 30 °C |
| Injection volume | 5 µl |
| Detector | UV-DAD<br>detection wavelength 260/40 nm,<br>reference wavelength 600/100 nm |

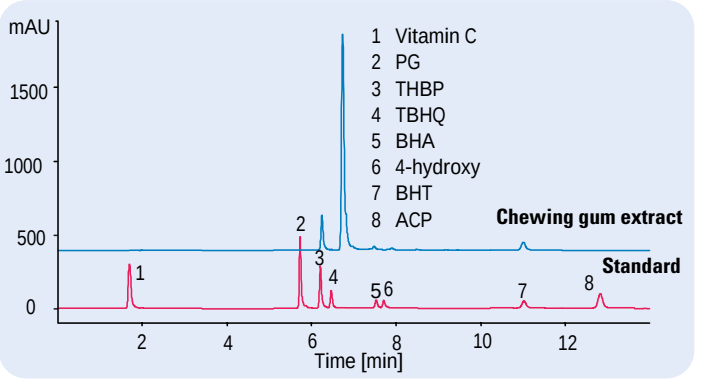
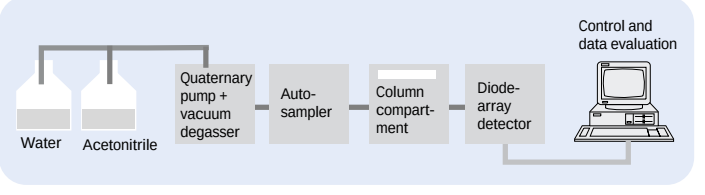



**Figure 4**
**Analysis of antioxidants in chewing gum**

**HPLC method performance**

| | |
|---|---|
| Limit of detection | 0.1–2 ng (injected amount), S/N = 2 |
| Repeatability of | |
| RT over 10 runs | < 0.2 % |
| areas over 10 runs | < 1 % |





*4. Official Methods of Analysis, Food Compositions; Additives, Natural Contaminants,* 15th ed; AOAC: Arlington, VA, **1990**, Vol. 2.;
AOAC Official Method 983.15: Antioxidants in oils and fats.

# Preservatives

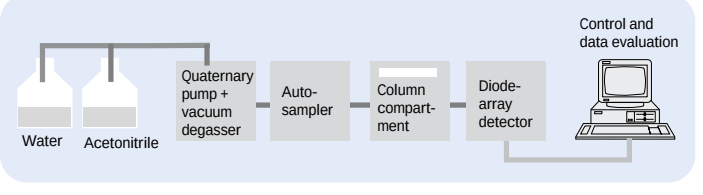
The following compounds are used as preservatives in food products:

- benzoic acid
- sorbic acid
- propionic acid
- methyl-, ethyl-, and propylesters of p-hydroxy benzoic acid (PHB-methyl, PHB-ethyl, and PHB-propyl, respectively)[4]

Preservatives inhibit microbial growth in foods and beverages. Various compound classes of preservatives are used, depending on the food product and the expected microorganism. PHBs are the most common preservatives in food products. In fruit juices, in addition to sulfur dioxide, sorbic and benzoic acid are used as preservatives, either individually or as a mixture.

### Sample preparation

Sample preparation depends strongly on the matrix to be analyzed. For samples low in fat, liquid extraction with ultrasonic bath stimulation can be used. For samples with more complex matrices, solid-phase extraction, liquid/liquid extraction, or steam distillation may be necessary.



## Chromatographic conditions

HPLC and UV-visible diode-array detection have been applied in the analysis of preservatives in white wine and salad dressing. Spectral information and retention times were used for identification.

| | |
|---|---|
| Sample preparation | Carrez clearing and filtration for the salad dressing. None for white wine. |
| Column | 125 x 4 mm Hypersil BDS, 5 µm |
| Mobile phase | A = water + 0.2 ml $H_2SO_4$, pH = 2.3<br>B = ACN |
| Gradient | start with 10 % B<br>at 3 min 60 % B<br>at 4 min 80 % B<br>at 6 min 90 % B<br>at 7 min 10 % B |
| Flow rate | 2 ml/min |
| Post time | 1 min |
| Column compartment | 40 °C |
| Injection volume | 2 µl |
| Detector | UV-DAD<br>detection wavelength 260/40 nm |

**HPLC method performance**

| | |
|---|---|
| Limit of detection | 10 ppm, S/N = 2 |
| Repeatability of | |
| RT over 10 runs | < 0.1 % |
| areas over 10 runs | < 3 % |

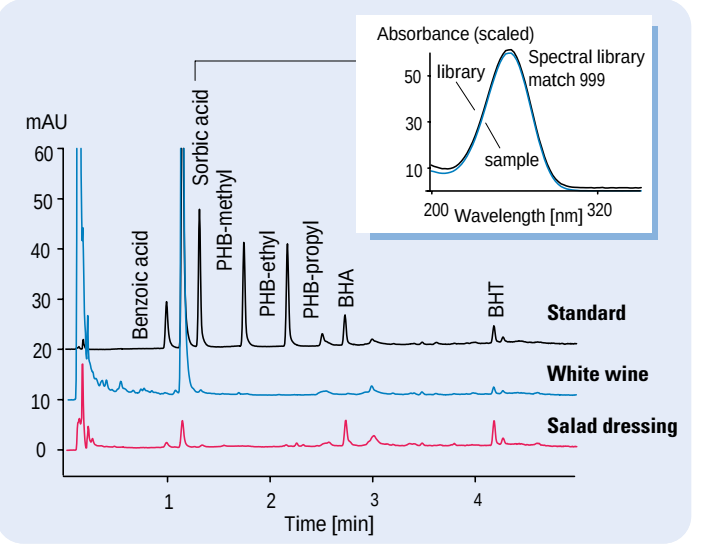



**Figure 5**
**Analysis of preservatives in white wine and salad dressing**


*4. Official Methods of Analysis, Food Compositions; Additives, Natural Contaminants,* 15th ed; AOAC: Arlington, VA, **1990**, Vol. 2.; AOAC Official Method 979.08: Benzoate, caffeine, saccharine in carbonated beverages.

## Artificial sweeteners

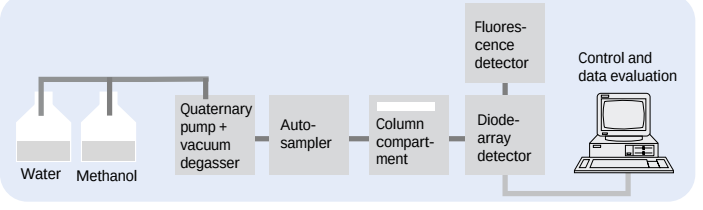
The following compounds are used as artificial sweeteners in food products:

- acesulfam
- aspartame
- saccharin[4]

Nowadays, low-calorie sweeteners are widely used in foods and soft drinks. Investigations of the toxicity of these compounds have raised questions as to whether they are safe to consume. As a result, their concentration in foods and beverages is regulated through legislation in order to prevent excessive intake.

### Sample preparation

Sample preparation depends strongly on the matrix to be analyzed. For sample low in fat, liquid extraction at low pH with ultrasonic bath stimulation can be used. For samples with more complex matrices, solid-phase extraction, liquid/liquid extraction, or steam distillation may be necessary.



## Chromatographic conditions

The HPLC method presented here for the analysis of aspartame is based on automated on-column derivatization and reversed-phase chromatography. UV spectra were evaluated as an additional identification tool.[5]

| | |
|---|---|
| Derivatization agent | o-phthalaldehyde (OPA) mercapto-propionic acid (MPA) |
| Column | 100 x 2.1 mm Hypersil ODS, 5 µm |
| Mobile phase | A = 0.01 mM sodium acetate<br>B = methanol |
| Gradient | start with 5 % B<br>at 5 min 25 % B<br>at 10 min 35 % B<br>at 13 min 55 % B<br>at 18 min 80 % B<br>at 20 min 95 % B |
| Flow rate | 0.35 ml/min |
| Post time | 5 min |
| Column compartment | 40 °C |
| Injection volume | 1 µl |
| Injector program for online derivatization | |
| 1. Draw 5.0 µl from vial 3 (borate buffer) | |
| 2. Draw 0.0 µl from vial 0 (water) | |
| 3. Draw 1.0 µl from vial 1 (OPA/MPA) | |
| 4. Draw 0.0 µl from vial 0 (water) | |
| 5. Draw 1.0 µl from sample | |
| 6. Mix 7 µl (6 cycles) | |
| 7. Inject | |
| Detectors | |
| UV-DAD: | detection wavelength 338/20 nm or |
| fluorescence: | excitation wavelength 230 nm, emission wavelength 445 nm |

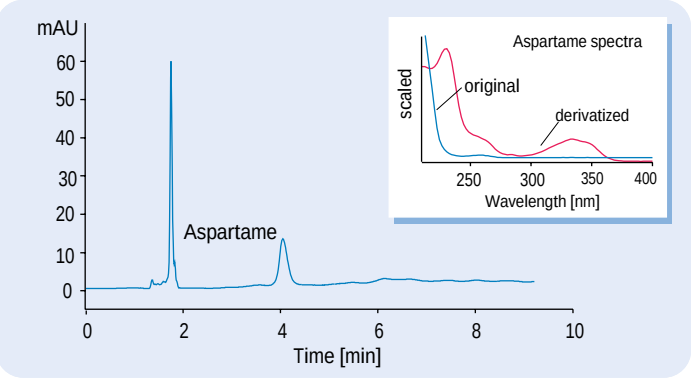



**Figure 6**
**Chromatogram and spectra of derivatized and non derivatized aspartame**

**HPLC method performance**

| | |
|---|---|
| Limit of detection | |
| for fluorescence | 200 pg (injected amount), S/N = 2 |
| for DAD | 1 ng (injected amount), S/N = 2 |
| Repeatability | |
| of RT over 10 runs | < 0.1 % |
| of areas over 10 runs | < 5 % |

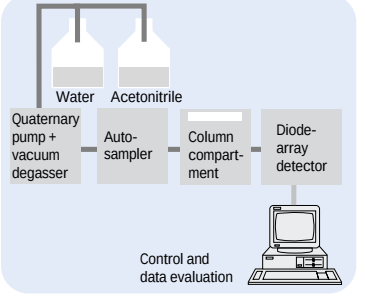

5. A.M. Di Pietra et al., "HPLC analysis of aspartame and saccharin in pharmaceutical and dietary formulations"; *Chromatographia*, **1990**, *30, 215–219.*
*4. Official Methods of Analysis, Food Compositions; Additives, Natural Contaminants,* 15th ed; AOAC: Arlington, VA, **1990**, Vol. 2.; Official Method *AOAC 979.08: Benzoate, caffeine, saccharin in soda beverages.*

## Colorants

We have selected the food color E104 Quinolin yellow and E131 Patent blue as application examples. Synthetic colors are widely used in the food processing, pharmaceutical, and chemical industries for the following purposes:[4]

- to mask decay
- to redye food
- to mask the effects of aging

The regulation of colors and the need for quality control requirements for traces of starting product and by-products have forced the development of analytical methods. Nowadays, HPLC methods used are based on either ion-pairing reversed-phase or ion-exchange chromatography.
UV absorption is the preferred detection method. The UV absorption maxima of colors are highly characteristic. Maxima start at approximately 400 nm for yellow colors, 500 nm for red colors, and 600–700 nm for green, blue, and black colors. For the analysis of all colors at maximum sensitivity and selectivity, the light output from the detector lamp should be high for the entire wavelength range. However, this analysis is not possible with conventional UV-visible detectors based on a one-lamp design. Therefore, we have chosen a dual-lamp design based on one deuterium and one tungsten lamp. This design ensures high light output for the entire wavelength range.




### Sample preparation

Whereas turbid samples require filtration, solid samples must be treated with 0.1 % ammonia in a 50 % ethanol and water mixture, followed by centrifugation. Extraction is then performed using the so-called wool-fiber method. After desorption of the colors and filtration, the solution can be injected directly into the HPLC instrument.

## Chromatographic conditions

The HPLC method presented here for the analysis of dyes is based on ion-pairing reversed-phase chromatography. UV spectra were evaluated as an additional identification tool.[6]

| | |
|---|---|
| Sample preparation | injection without further preparation |
| Column | 125 x 3 µm Hypersil BDS, 3 mm |
| Mobile phase | A = 0.01 M $NaH_2PO_4$+ 0.001 M tetrabutyl-ammoniumdihydrogen-phosphate, pH = 4.2<br>B = ACN |
| Gradient | start with 15 %<br>in 10 min to 40 %<br>in 14 min to 90 %<br>until 19 min at 90 %<br>in 20 min to 15 % ACN |
| Stop time | 20 min |
| Post time | 4 min |
| Flow rate | 0.8 ml/min |
| Column compartment | 40 °C |
| Injection volume | 1 µl |
| Detector | UV-DAD<br>signal A: 254/50 nm (for optimization of separation)<br>signal B: 350/20 nm<br>signal C: 465/30 nm<br>signal D: 600/40 nm |

**HPLC method performance**

| | |
|---|---|
| Limit of detection for UV-DAD | 2 ng (injected amount) S/N = 2 |
| Repeatability of RT over 10 runs | < 0.2 % |
| of areas over 10 runs | < 3 % |

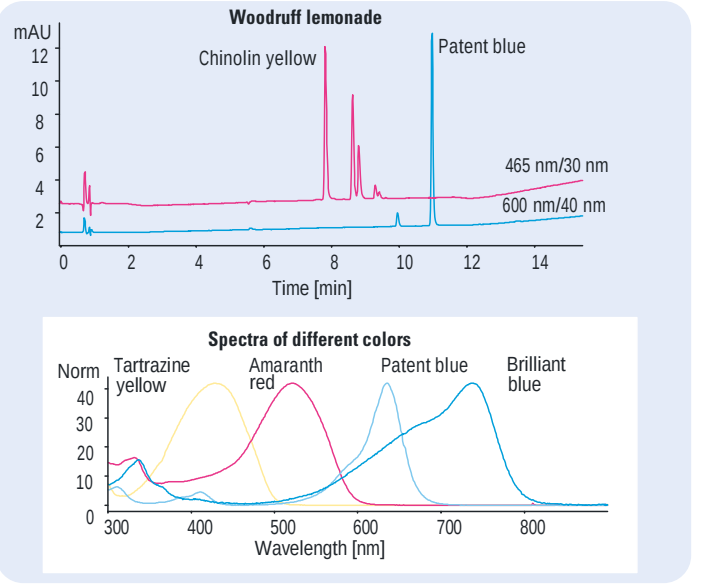



**Figure 7**
**Analysis of synthetic colors in lemonade. Overlay of spectra of yellow, red, blue and "black" colors**


4. *Official Methods of Analysis, Food Compositions; Additives, Natural Contaminants,* 15th ed; AOAC: Arlington, VA, **1990**, Vol. 2.; Official Method AOAC 981.13: Cresidine sulfonic acid in FD&C Red No. 40; Official Method AOAC 982.28: Intermediates and reaction by-products in FD&Y Yellow No. 5; Official Method AOAC 977.23: 44' (Diazoamino) dibenzene sulfonic acid (DAADBSA) in FD&C Yellow No. 6; Official Method AOAC 980.24: Sulfanilic acid in FD&C Yellow No. 6.

6. A.G. Huesgen, R.Schuster, "Sensitive analysis of synthetic colors using HPLC and diode-array detection at 190–950 nm", *Agilent Application Note 5964-3559E,* **1995**.

# Flavors

The following compounds are examples of flavoring agents used in food products:

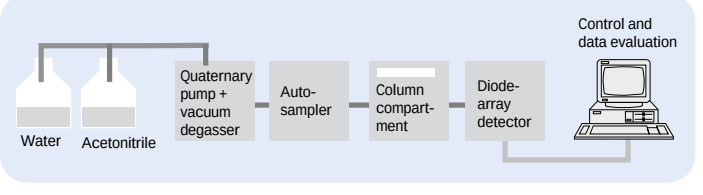
- lupulon and humulon (hop bittering compounds)
- vanillin
- naringenin and hesperidin (bittering compounds)

Three major classes of compounds are used as flavoring agents: essential oils, bitter compounds, and pungency compounds. Although the resolution afforded by gas chromatography (GC) for the separation of flavor compounds remains unsurpassed, HPLC is the method of choice if the compound to be analyzed is low volatile or thermally unstable.

## Vanillin

### Sample preparation

Turbid samples require filtration, whereas solid samples must be extracted with ethanol. After filtration, the solution can be injected directly into the HPLC instrument.



| | |
|---|---|
| Sample preparation | injection without further preparation |
| Column | 100 x 4 mm Hypersil BDS, 3 µm |
| Mobile phase | A = water + 0.15 ml $H_2SO_4$ (conc.), pH = 2.3<br>B = ACN |
| Gradient | start with 10 % B<br>at 3 min 40 % B<br>at 4 min 40 % B<br>at 6 min 80 % B<br>at 7 min 90 % B |
| Flow rate | 0.8 ml/min |
| Post time | 3 min |
| Column compartment | 30 °C |
| Injection volume | 5 µl |
| Detector | UV-DAD<br>detection wavelength 280/80 nm,<br>reference wavelength 360/100 nm |

## Chromatographic conditions

The HPLC method presented here for the analysis of vanillin is based on reversed-phase chromatography. UV spectra were evaluated as an additional identification tool.[7]

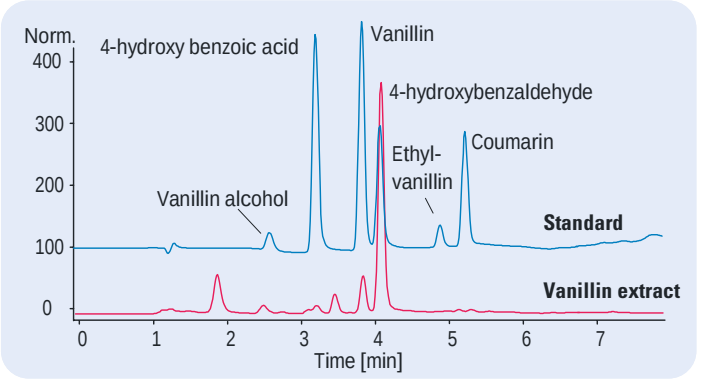



**Figure 8**
**Determination of the quality of vanillin extract**

| | |
|---|---|
| Conditions as above, except | |
| Column | 100 x 2.1 mm Hypersil ODS, 5 µm |
| Mobile phase | A = water + 5 mM $NaH_2PO_4$<br>B = methanol |
| Gradient | at 10 min 70 % B |
| Flow rate | 0.4 ml/min |

**HPLC method performance**

| | |
|---|---|
| Limit of detection | 0.2–5 ng (injected amount) S/N = 2 |
| Repeatability | |
| of RT over 10 runs | < 0.2 % |
| of areas over 10 runs | < 1 % |

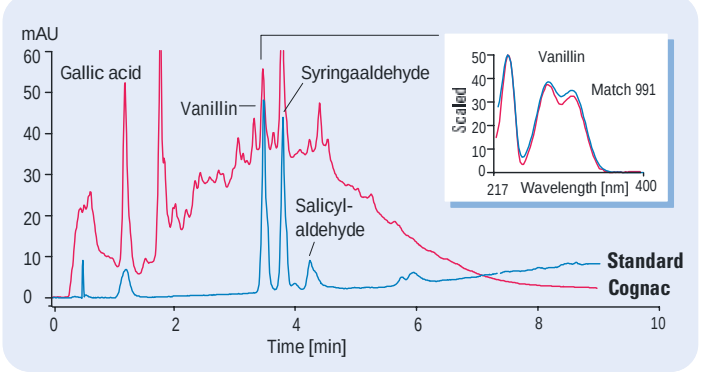



**Figure 9**
**Analysis of vanillin in cognac. Identification of vanillin through spectra comparison**


7. Herrmann, A, et al.;„"Rapid control of vanilla-containing products using HPLC"; *J. Chromatogr.*, **1982**, 246, 313–316.

# Bitter compounds: hesperidin and naringenin

## Sample preparation for bitter compounds in orange juice[8]

The samples were prepared according to Carrez 1 and 2. This method uses potassium ferrocyanide and zinc sulfate for protein precipitation.

## Chromatographic conditions

The HPLC method presented here for the analysis of hesperidin and naringenin is based on reversed-phase chromatography. UV spectra were evaluated as an additional identification tool.

| | |
|---|---|
| Sample preparation | The orange juice was prepared according to Carrez 1 and 2. |
| Column | 125 x 4 mm Hypersil BDS, 5 µm |
| Mobile phase | A = water + 0.15 ml/l $H_2SO_4$ (conc.), pH = 2.4<br>B = ACN |
| Gradient | start with 20 % B<br>at 3 min 20 % B<br>at 5 min 90 % B<br>at 6 min 20 % B |
| Flow rate | 2 ml/min |
| Post time | 1 min |
| Column compartment | 40 °C |
| Injection volume | 1 µl |
| Detector | UV-DAD detection wavelength 260/80 nm, reference wavelength 380/80 nm |

**HPLC method performance**

| | |
|---|---|
| Limit of detection for DAD | 1 ng (injected amount), S/N = 2 |
| Repeatability of RT over 10 runs | < 0.2 % |
| of areas over 10 runs | < 1 %. |

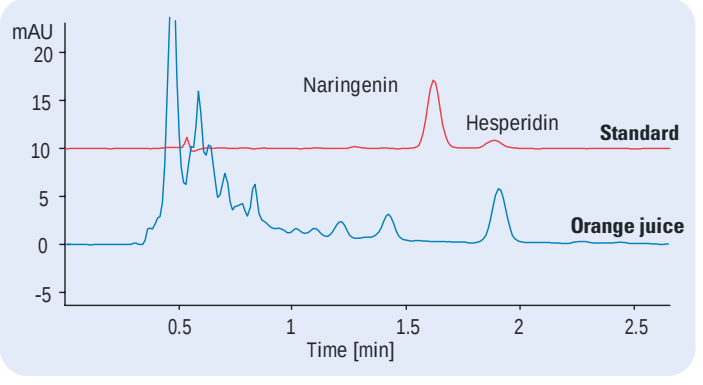
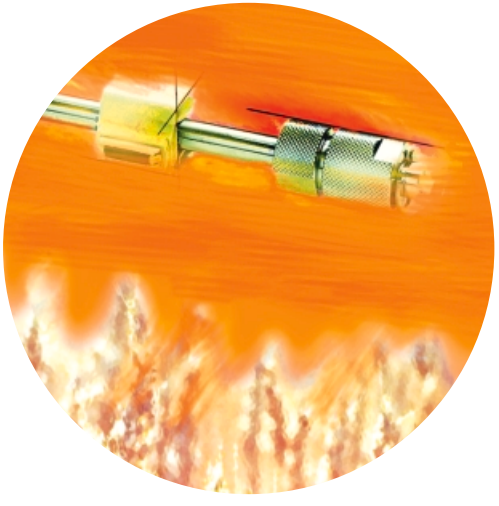



**Figure 10**
**Analysis of bitter compounds in orange juice**

8. *Official Methods of Analysis*; Horwitz, W., Ed.; 14th ed.; AOAC: Arlington, VA, **1984**; secs 12.018–12.021.

*Chapter 2*

# Analytical examples of residues and contaminants



2

# Residues of chemotherapeutics and antiparasitic drugs

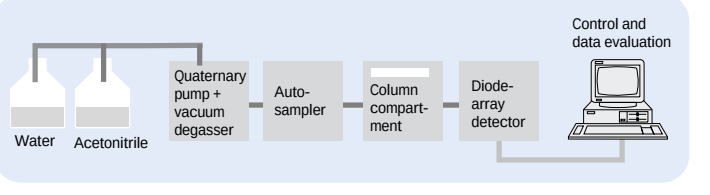
In addition to several other drugs, nitrofurans and sulfonamides such as sulfapyridine, N-acetyl metabolite, ethopabat, chloramphenicol, meticlorpindol, metronidazol, ipronidazol, furazolidone, and nicarbazin are frequently fed to domestic cattle.

Modern intensive animal breeding demands permanent suppression of diseases caused by viruses, bacteria, protozoa, and/or fungi. A number of chemotherapeutics are available for the prevention and control of these diseases. After application, residues of these drugs can be found in foods of animal origin such as milk, eggs, and meat. These chemotherapeutics can cause resistancy of bacteria. Because of the toxic nature of chemotherapeutics, for example, choramphenical, government agencies in many countries, including the United States, Germany, and Japan, have set tolerance levels for residues of these drugs.

Simple and reliable analysis methods are necessary in order to detect and quantify residues of chemotherapeutic and antiparasitic drugs in food products. Malisch et al. have developed an HPLC method to determine 11 of these compounds.[9,10] The internal standard (ISTD) comprises benzothiazuron and pyrazon.

### Sample preparation

After homogenization or mincing and pH adjustment, the samples were extracted using liquid/liquid extraction followed by degreasing, purification, and concentration.



| | |
|---|---|
| Sample preparation | Sample preparation was done according to reference[9] |
| Column | 250 x 4.6 mm Spherisorb ODS-2, 5 µm |
| Mobile phase | A = sodium acetate buffer, 0.02 M, pH = 4.8<br>B = ACN/water (60:40) |
| Gradient | start with 8 % B<br>at 5 min 8 % B<br>at 7 min 20 % B<br>at 14 min 23 % B<br>at 16 min 33 % B<br>at 19 min 40 % B<br>at 21 min 50 % B<br>at 26 min 60 % B<br>at 30 min 80 % B<br>at 33 min 90 % B<br>at 43 min 90 % B<br>at 55 min 8 % B |
| Flow rate | 1.5 ml/min |
| Injection volume | 20 µl |
| Detector | UV-DAD<br>detection wavelengths 275/80 nm, 315/80 nm, and 360/80 nm, reference wavelength 500/100 nm |

**HPLC method performance**

| | |
|---|---|
| Limit of detection | 0.001–0.05 mg/kg |
| Repeatability | |
| of RT over 10 runs | < 0.12 % |
| of areas over 10 runs | < 1.5 % |

## Chromatographic conditions

The HPLC method presented here for the analysis of residues of drugs in eggs, milk, and meat is based on reversed-phase chromatography and multisignal UV-visible diode-array detection (UV-DAD). UV spectra were evaluated as an additional identification tool.

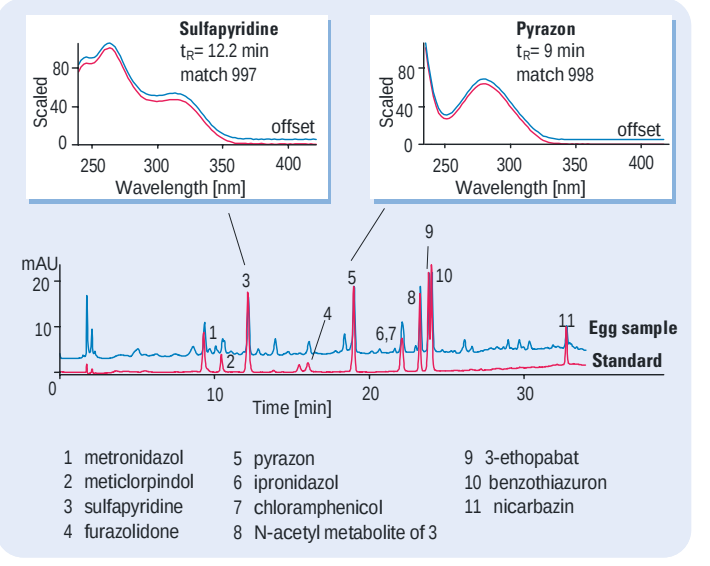



**Figure 11**
**Analysis of residues in an egg sample. Identification through spectra comparison**


9. H. Malisch, et al.,"Determination of residues of chemotherapeutic and antiparasitic drugs in food stuffs of anomaly origin with HPLC and UV-Vis diode-array detection", *J. Liq. Chromatogr.,* **1988**, 11 (13), 2801–2827.14.
10. EC Guideline 86/428 EWG **1985**.

2

## Tetracyclines

Tetracyclines are used worldwide as oral or parenteral medication in the form of additives in animal feed. In food-producing animals, these drugs exhibit a high degree of activity toward a wide range of bacteria.[9, 11]

### Sample preparation

After homogenization or mincing and addition of mineral acids to dissociate tetracyclines from proteins, the samples were extracted using liquid/liquid extraction followed by degreasing and/or deproteinization, purification, and concentration.[12]

### Chromatographic conditions

The HPLC method presented here for the analysis of meat is based on reversed-phase chromatography and UV-visible diode-array detection. UV spectra were evaluated as an additional identification tool.

| | |
|---|---|
| Sample preparation | 1 g sample was mixed with citric acid (100 mg).<br>➔ add 1 ml nitric acid (30 %) or 0.1 m oxalic acid<br>➔ add 4 ml methanol 5 min in the ultrasonic bath<br>➔ add water up to 10 ml total volume<br>➔ centrifuge<br>➔ inject |
| Column | 100 × 4 mm Hypersil BDS, 3 µm |
| Mobile phase | A = water, pH = 2.1 with sulfuric acid<br>B = ACN |
| Gradient | start with 15 % B<br>at 10 min 60 % B |
| Flow rate: | 0.5 ml/min |
| Column compartment | 25 °C |
| Detector | UV-DAD<br>detection wavelength 355 nm/20 nm,<br>reference wavelength 600/100 nm |

**HPLC method performance**

| | |
|---|---|
| Limit of detection for UV-DAD | 100 ppb |
| Repeatability of RT over 10 runs | < 0.2 % |
| of areas over 10 runs | < 2 % |

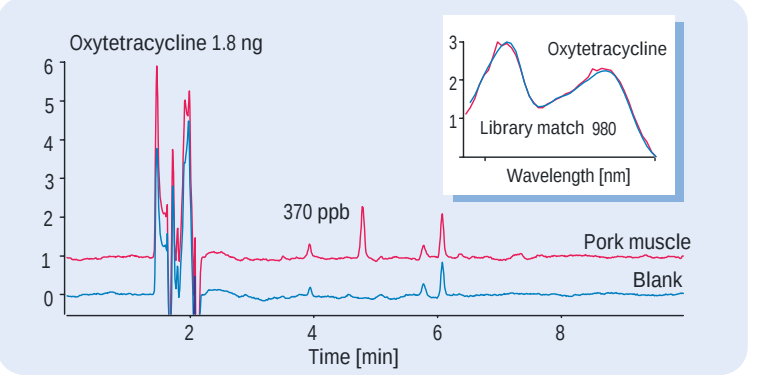



**Figure 12**
**Trace analysis of tetracycline residues in meat. Identication of oxytetracycline through spectra comparison**


9. H. Malisch et al., "Determination of residues of chemotherapeutic and antiparasitic drugs in food stuffs of anomaly origin with HPLC and UV-Vis diode-array detection" *J. Liq. Chromatogr.*, **1988**, 11 (13), 2801–2827.14.
11. M.H. Thomas, *J. Assoc. Off. Anal.;* **1989** , 72 (4) 564.
12. Farrington et. al., "Food Additives and Contaminants, **1991**, Vol. 8, No. 1, 55-64".

## Fumonisins

Fumonisins are characterized by a 19-carbon aminopoly-hydroxyalkyl chain which is diesterified with propane-1,2,3-tricarboxylic acid. Analogues B 1-3 in figure 13 show a difference only in the number and position of the hydroxyl groups present on the molecule.

Fragmentation experiments using collision induced dissociation (CID) show no difference between fumonisins B2 and B3. Consequently, it was necessary to separate these compounds chromatographically for quantitative analysis. However, in crude corn extracts the CID-fragment ions provide important confirmatory information. In order to obtain spectra of the fragment ions as well as the pseudo-molecular ions in a single scan, operating at maximum sensitivity, the fragmentor voltage was set to 230 V while scanning from 150 amu to 680 amu and then to 100 V when scanning from 690 amu to 800 amu.

### Sample preparation

Extraction according to § 35, LMBG.[13]

### Chromatographic conditions

The Agilent 1100 Series LC/MSD proved to be capable of detecting and quantifying fumonisins at 250 picograms per component regardless of their chemical structure and without the need for derivatization during the sample preparation procedure. The Agilent 1100 Series LC/MSD provided optimum sensitivity in the selected ion monitoring mode. Even when operating in scan mode (150 amu to 800 amu), the Agilent 1100 Series LC/MSD still provided sensitivity more than a factor of 10 better than reported for a fluorescence detector.

**LC/MS conditions**

| | |
|---|---|
| Colum | Zorbax Eclipse XDB-C18, 2.1 mm x 150 mm, 5 µm |
| Mobile phase A | 5 mM ammonium acetate pH3 |
| Mobile phase B | acetonitrile |
| Gradient | 0 min 33% B<br>8 min 60% B<br>9 min 33% B |
| Flow rate | 250 µl/min |
| Injection vollume | 5 µl |
| Column compartment | 40°C |
| Ionization mode | API-ES positive or APCI negative |
| Nebulizer pressure | 30 psig |
| Dryng gas temp. | 350°C |
| Drying gas flow | 6 l/min |
| Vcap. | 4000 volts |
| Fragmentor | 100 volts |
| Scan range | m/z 120 –820 |

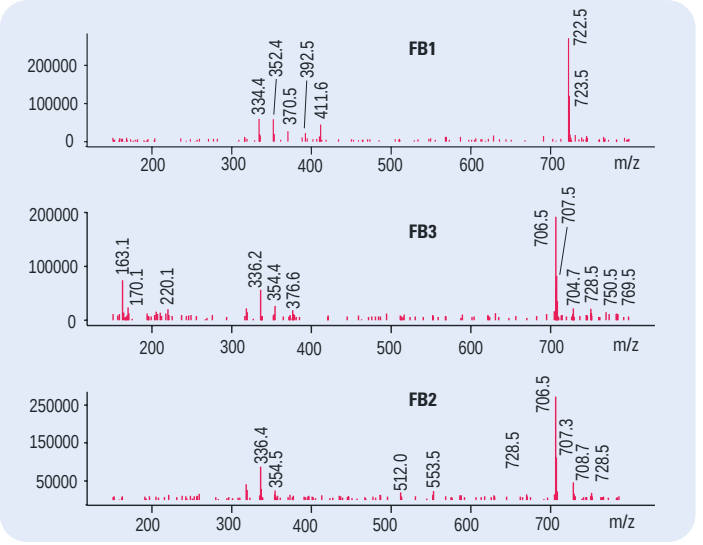



**Figure 13**
**Mass spectra of Fumonisins B 1,2,3 when the fragmentor is ramped from 230 to 100V**

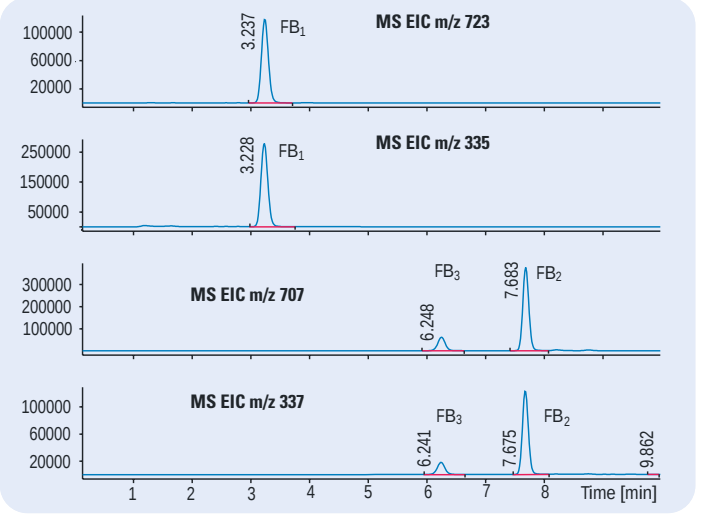



**Figure 14**
**Identification of different Fumonisin species in corn extract by retention time with further confirmation through fragment ion**

13. Lebensmittel- und Bedarfsgegenständegesetz, Paragraph 35, Germany.

## Mycotoxins

The following mycotoxins have been analyzed: aflatoxins $G_2$, $G_1$, $B_2$, $B_1$, $M_2$, and $M_1$; ochratoxin A; zearalenone; and patuline.

Mycotoxins are highly toxic compounds produced by fungi. They can contaminate food products when storage conditions are favorable to fungal growth. These toxins are of relatively high molecular weight and contain one or more oxygenated alicyclic rings. The analysis of individual mycotoxins and their metabolites is difficult because more than 100 such compounds are known, and any individual toxin is likely to be present in minute concentration in a highly complex organic matrix. Most mycotoxins are assayed with thin-layer chromatography (TLC). However, the higher separation power and shorter analysis time of HPLC has resulted in the increased use of this method. The required detection in the low parts per billion (ppb) range [4,13] can be performed using suitable sample enrichment and sensitive detection.

### Sample preparation

Samples were prepared according to official methods.[13] Different sample preparation and HPLC separation conditions must be used for the different classes of compounds. The table on the next page gives an overview of the conditions for the analysis of mycotoxins in foodstuffs.

### Chromatographic conditions

The HPLC method presented here for the analysis of mycotoxins in nuts, spices, animal feed, milk, cereals, flour, figs, and apples is based on reversed-phase chromatography, multisignal UV-visible diode-array detection, and fluorescence detection. UV spectra were evaluated as an additional identification tool.

| Column class | Matrix | Sample preparation | Chromatographic conditions |
|---|---|---|---|
| **Aflatoxins** $G_2$, $G_1$, $B_2$, $B_1$, $M_2$, $M_1$ | nuts, spices, animal feed, milk, dairy products | ⇨ extraction according to Para. 35, LMBG*[8,12] | Hypersil ODS, 100 × 2.1 mm id, 3-µm particles<br>water/methanol/ACN (63:26:11) as isocratic mixture**<br>flow rate: 0.3 ml/min at 25 °C<br>DAD: 365/20 nm<br>Fluorescence detector (FLD): excitation wavelength 365 nm, emission wavelength 455 nm |
| **Ochratoxin A** | cereals, flour, figs | ⇨ extraction according to Para. 35, LMBG<br>⇨ acidify with HCl<br>⇨ extract with toluene<br>⇨ $SiO_2$ cleanup elute toluene/acetic acid (9:1) | Lichrospher 100 RP18, 125 × 4 mm id, 5-µm particles<br>water with 2 % acetic acid/ACN (1:1)*<br>flow rate: 1 ml/min at 40 °C<br>FLD: excitation wavelength 347 nm, emission wavelength 480 nm |
| **Zearalenone** | cereals | ⇨ extract with toluene<br>⇨ Sep-pak cleanup<br>⇨ elute toluene/acetone (95:5)<br>⇨ AOAC 985.18:4 α-zearalenol and zearalenone in corn | Hypersil ODS, 100 × 2.1 mm id, 3 µm particles<br>water/methanol/ACN (5:4:1) isocratic mixture*<br>flow rate: 0.45 ml/min at 45 °C<br>DAD: 236/20 nm<br>FLD: excitation wavelength 236 nm, emission wavelength 464 nm |
| **Patuline** | apple products | ⇨ cleanup on Extrelut<br>⇨ silica gel cleanup<br>⇨ elute toluene/ethylacetate (3:1) | Superspher RP18, 125 × 4 mm id, 4-µm particles<br>water 5 %–95 % ACN<br>flow rate: 0.6 ml/min at 40 °C<br>DAD: 270/20 nm<br>or<br>Lichrospher diol, 125 × 4 mm id, 5-µm particles<br>hexane/isopropanol (95:5) as isocratic mixture<br>flow rate: 0.6 ml/min at 30 °C<br>DAD: 270/20 nm |

* Lebensmittel- und Bedarfsgegenständegesetz, Germany

** 100 % B is recommended for cleaning the column

**HPLC method performance**

| | |
|---|---|
| Limit of detection | 1–5 µg/kg |
| Repeatability | |
| of RT over 10 runs | < 0.12 % |
| of areas over 10 runs | < 1.5 % |
| Linearity | |
| of UV-visible DAD | 1–500 ng |
| of fluorescence | 30 pg to 2 ng |

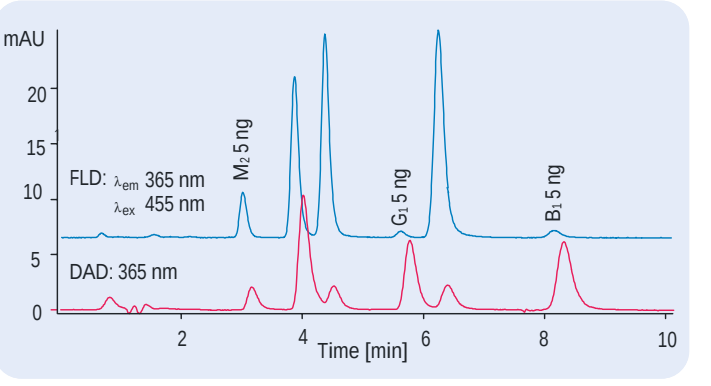



**Figure 15**
**Analysis of aflatoxins with UV and fluorescence detection**

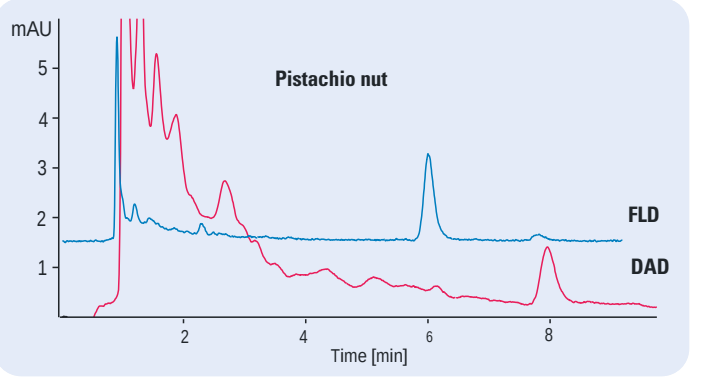
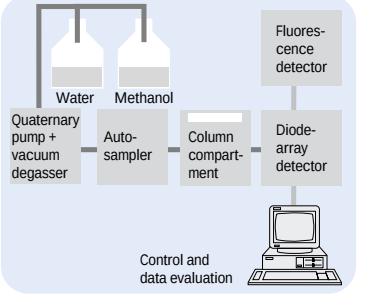



**Figure 16**
**Analysis of aflatoxins in pistachio nuts with UV and fluorescence detection**




13. Lebensmittel- und Bedarfsgegenständegesetz, Paragraph 35, Germany. 4. *Official Methods of Analysis, Food Compositions; Additives, Natural Contaminants*, 15th ed; AOAC: Arlington, VA, **1990**, Vol. 2.; AOAC Official Method 980.20: aflatoxins in cotton seed products; AOAC Official Method 986.16: Aflatoxins $M_1$ , $M_2$ in fluid milk; AOAC Official Method 985.18: α-zearalenol.

# Bisphenol A diglycidyl-ether (BADGE)

Bisphenol A diglycidyl-ether (BADGE) is present in the three most common coatings (epoxy lacquer, organosol lacquer and polyester lacquer) used to protect the inside surfaces of cans used for food packaging. In canned foods containing a high proportion of fat, BADGE tends to migrate into the fatty phase where it remains stable, whereas in water it is hydrolyzed.

BADGE was originally determined to be mutagenic during in vitro tests but a later re-assessment, using in vivo tests, led to a different conclusion. While further tests are being performed, a maximum concentration of 1 mg BADGE per kg of food has been agreed.

### Sample preparation

Extracted with water/alcohol 50/50 or n-heptane at reflux temperature for six hours.

### Chromatographic conditions

A fast separation was developed by using the enhanced specificity provided by the Agilent 1100 Series LC/MSD in CID (collision induced dissociation) mode allowing the detection of BADGE via the molecular ion combined with confirmation using the most abundant fragment ion.

**LC/MS conditions**

| | |
|---|---|
| Colum | Zorbax Eclipse XDB-C8, 2.1 mm x 50 mm, 5 µ |
| Mobile phase A | 5 mM ammonium acetate in water, pH3 |
| Mobile phase B | acetonitrile |
| Gradient | 0 min 25% B<br>5 min 50 % B |
| Flow rate | 300 µl/min |
| Injection volume | 1 µl |
| Column compartment | 40 °C |
| Detector | UV-DAD<br>210 nm/6 nm, ref. 360/60 nm<br>254 nm/6 nm, ref. 360/60 nm |

| | |
|---|---|
| Ionization mode | API-ES positive |
| Nebulizer pressure | 50 psig |
| Dryng gas temp. | 350 °C |
| Vcap. | 3500 volts |
| Fragmentor | 70 volts |
| Scan range | m/z 250 –400 |
| Scan speed | 2 s/scan |

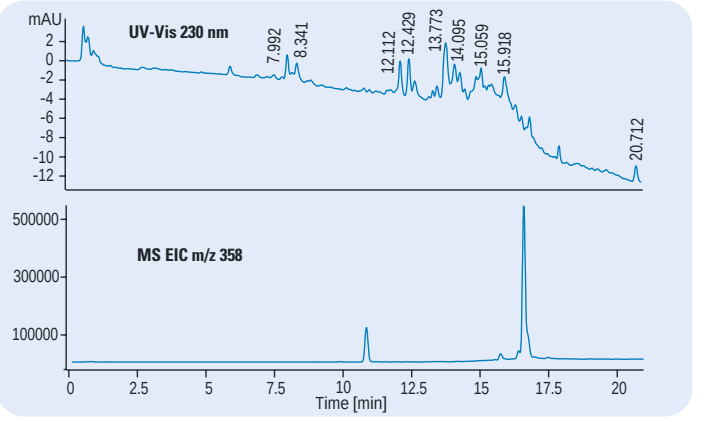



**Figure 17**
**Extract from tuna 0.2 ppm, 1 µl injected**

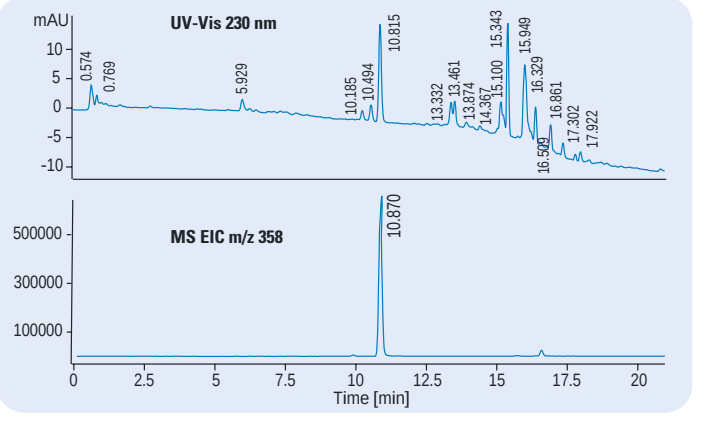


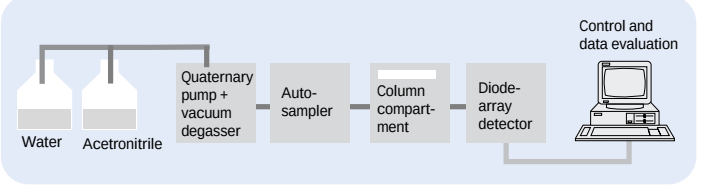

**Figure 18**
**Extract from sardine 20 ppm, 1 µl injected**

## Pesticides

The following compound classes of pesticides have been analyzed: triazines, phenylurea-herbicides, methabenzthiazuron, diquat, paraquat, and mercaptobenzothiazol. Carbamates and glyphosate also have been analyzed but with different equipment. In most countries, growing concern about the residues of pesticides in food products is evident. Therefore, regulations limiting the concentration of pesticides in foodstuffs have been introduced to protect consumers from contaminated food products. Several methods are used to control these limits. HPLC is recommended for the analysis of low volatile compounds and for compounds that are unstable when heated.

### Sample preparation

Sample preparation and enrichment depend strongly on the matrix. Drinking water samples, for example, must be extracted using solid-phase extraction, whereas vegetables are extracted with liquid/liquid extraction after homogenization, followed by additional cleaning and sample enrichment.




14. Specht, W. “Organochlor- und Organophosphor-Verbindungen sowie stickstoffhaltige sowie andere Pflanzenschutzmittel”, DFG-Methodensammlung, **1982**, 19.

## Chromatographic conditions

The HPLC method presented here was used for the analysis of pesticides in salad samples and spices.

| | |
|---|---|
| Sample preparation | Salad was homogenized and then extracted with liquid/liquid extraction. The extract was cleaned with gel permeation chromatography using cyclohexane/ethyl acetate. Spices were prepared according to Specht[14] with gel permeation chromatography. |
| Column | 100 × 3 mm Hypersil BDS, 3 µm |
| Mobile phase | water/ACN (95:5) |
| Gradient | at 10 min 25 % ACN<br>at 26 min 42 % ACN<br>at 34 min 60 % ACN |
| Flushing time | 10 min at 100 % ACN |
| Post time | 6 min |
| Flow rate | 0.5 ml/min |
| Oven temperature | 42 °C |
| Injection volume | 3–10 µl |
| Detector | UV-DAD<br>detection wavelengths 214/15 nm, 230/20 nm, and 245/20 nm<br>reference wavelength 400/80 nm |

**HPLC method performance**

| | |
|---|---|
| Limit of detection | 0.01 µg/l |
| Repeatability | |
| of RT over 10 runs | < 0.2 % |
| of areas over 10 runs | < 1 % |

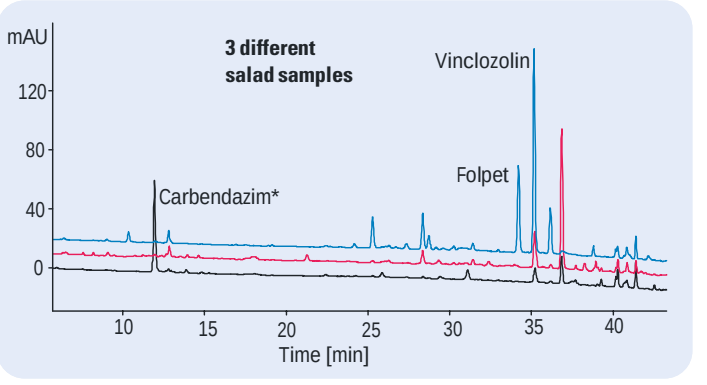



**Figure 19**
**Analysis of pesticide residues in three different salad samples**
* Carbendazim has a low recovery rate of only approximately 40 %

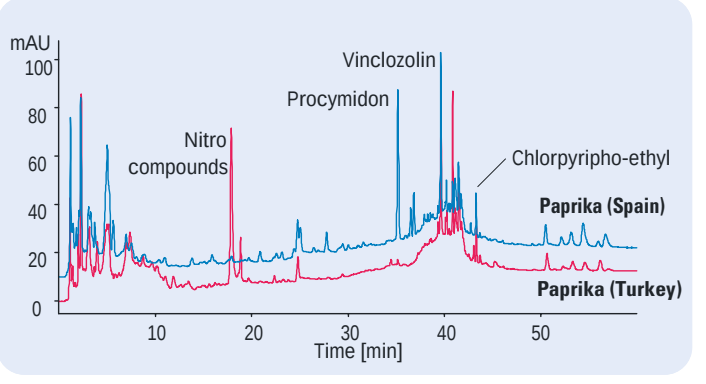


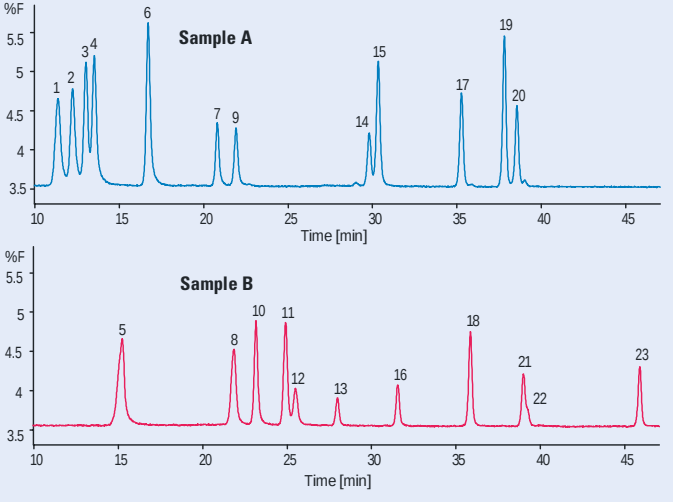

**Figure 20**
**Analysis of pesticide residues in two paprika samples**

# Carbamates

## Chromatographic conditions

The HPLC method presented here was used for the direct analysis of carbamates in water with postcolumn derivatization.[15] Fruits and vegetables must be extracted at neutral pH with water prior to HPLC analysis.

| | |
|---|---|
| Sample preparation | none |
| Column | 250 x 4 mm C18 phase from Pickering, 5 µm |
| Mobile phase | water/methanol (MeOH, 88:12) |
| Gradient | at 2 min 12 % MeOH<br>at 42 min 66 % MeOH<br>at 46 min 66 % MeOH<br>at 46.1 min 100 % MeOH<br>at 49 min 100 % MeOH |
| Flow rate | 0.8 ml/min |
| Column compartment | 37 °C |
| Injection volume | 10 µl standard |
| Fluorescence detector | |
| Excitation wavelength: 230 nm or 330 nm | |
| Emission wavelength: 425 nm | |
| Photomultiplier gain: 12 | |
| Response time: 4 s | |
| Derivatization reagent pump | |
| flow rate for hydrolization agent: 0.3 ml/min (NaOH) | |
| flow rate for derivatization agent: 0.3 ml/min (OPA) | |




**Sample A**

1 butocarboxim sulfoxide
2 aldicarb sulfoxide
3 butoncarboxim sulfone
4 aldicarb sulfone
6 methomyl
7 ethiofencarb sulfoxide
9 ethiofencarb sulfone
14 butocarboxim
15 aldicarb
17 propoxur
19 carbaryl
20 ethiofencarb

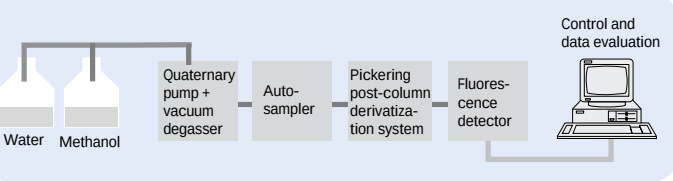
**Sample B**

5 oxamyl
8 thiofanox sulfoxide
10 thiofanox sulfone
11 3-hydroxycarbofuran
12 methiocarb sulfoxide
13 methiocarb sulfone
16 3-ketocarbofuran
18 carbofuran
21 1-naphthol
22 thiofanox
23 methiocarb

**Figure 21**
**Analysis of two different carbamate standards**

**HPLC method performance**

| | |
|---|---|
| Limit of detection | 100 ppt, S/N = 2 |
| Repeatability | |
| of RT over 10 runs | < 0.1 % |
| of areas over 10 runs | < 0.5–5 % |




15. "A new approach to lower limits of detection and easy spectral analysis" *Agilent Primer 5968-9346E*, **2000**

# Glyphosate

## Chromatographic conditions

The HPLC method presented here was used for the direct analysis of glyphosate in water with postcolumn derivatization.[16]

| | |
|---|---|
| Sample preparation | none |
| Column | 150 x 4 mm cation exchange, K + form from Pickering, 8 µm |
| Mobile phase | A = 5 mM $KH_2PO_4$, pH = 2.0<br>B = 5 mM KOH |
| Flow rate | 0.4 ml/min |
| Gradient | at 15 min 0 % B<br>at 17 min 100 % B |
| Column compartment | 55 °C |
| Injection volume | 50 µl standard |
| Fluorescence detector | |
| Excitation wavelength: 230 nm or 330 nm | |
| Emission wavelength: 425 nm | |
| Photomultiplier gain: 12 | |
| Response time: 4 s | |
| Derivatization reagent pump | |
| flow rate for hydrolization agent: 0.3 ml/min (OCl*) | |
| flow rate for derivatization agent: 0.3 ml/min (OPA) | |

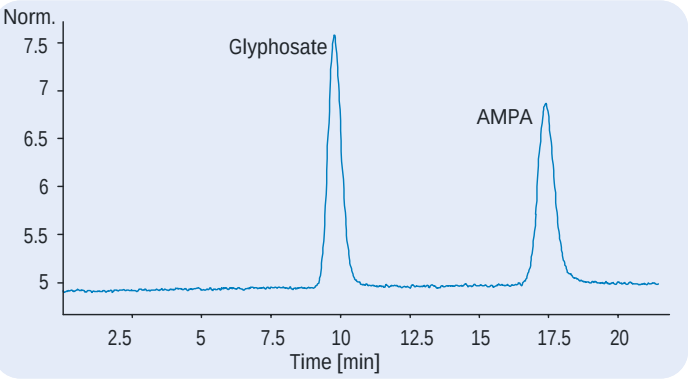
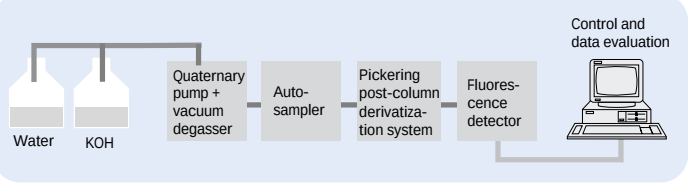



**Figure 22**
**Analysis of glyphosate standard**

**HPLC method performance**

| | |
|---|---|
| Limit of detection | 500 ppt |
| Repeatability | |
| of RT over 10 runs | < 0.8 % |
| of areas over 10 runs | < 2.2 % |




16. R. Schuster, “A comparison of pre- and post-column sample treatment for the analysis of glyphosate”, *Agilent Application Note 5091-3621E* , **1992**.

*Chapter 3*

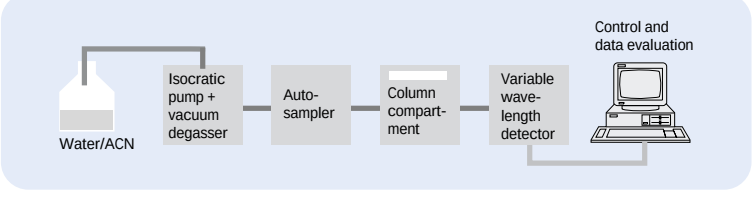
# Analytical examples of natural components

# Inorganic anions

Anions containing halogen, nitrogen, and sulfur are used as additives in food industries. For example, nitrites act as preservatives in smoked sausage. Nowadays, dedicated instrumentation such as special columns and electro-conductivity detectors are used in the analysis of inorganic anions. Because specialized equipment has a very limited application range, a method was developed for analyzing anionsusing reversed-phase chromatography and indirect UV detection. Another, more selective and sensitive approach for the analysis of selected anions is electro-chemical detection.

## Sample preparation

Excepting filtration, sample preparation normally is unnecessary if the sample is aqueous. Other matrices can be extracted with hot water, followed by filtration.



## Chromatographic conditions

The HPLC method presented here was used for the analysis of anions in drinking water.

| | |
|---|---|
| Sample preparation | filtration |
| Column | HP-IC (modifiers for the mobile phase are included) |
| Mobile phase | water/acetonitrile (ACN) (86:14), adjusted to pH = 8.6 with carbonate-free NaOH |
| Flow rate | 1.5 ml/min |
| Oven temperature | 40 °C |
| Injection volume | 25 µl |
| Detector | UV-VWD detection wavelength 266 nm |

**HPLC method performance**

| | |
|---|---|
| Limit of detection for UV-VWD | 0.1–1 ppb with S/N = 2 and 25 µl injected volume |
| Repeatability of | |
| RT over 10 runs | < 0.8 % |
| areas over 10 runs | < 1 % |

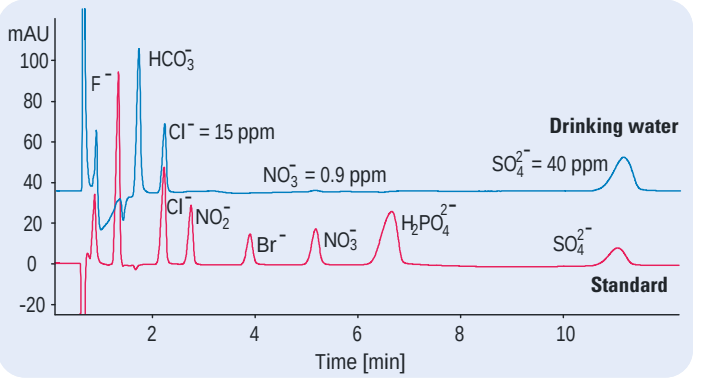



**Figure 23**
**Analysis of anions in drinking water with indirect UV-detection**

## Chromographic conditions for electrochemical detection

The HPLC method presented here was used for the analysis of iodide in table salt.[17]

| | |
|---|---|
| Sample preparation | Table salt was dissolved in water. |
| Column | 200 x 4 mm Sperisorb ODS2, 5 µm |
| Mobile phase | water with 5.2 g/l $K_2HPO_4$ + 3 g/l tetrabutylammoniumdi-hydrogenphosphat/ACN (85:15) |
| Flow rate | 1 ml/min |
| Oven temperature | ambient 24 °C |
| Injection volume | 0.1 µl |
| Detector | electrochemical (ECD) |
| Electrode: | glassy carbon, |
| Working potential: | 1 V |
| Operation mode: | amperometry |

**HPLC method performance**

| | |
|---|---|
| Limit of detection for ECD | 40 µg/l |
| Repeatability of RT over 10 runs | < 0.1 % |
| areas over 10 runs | 3 % |
| Linearity | min 50 pg to 150 ng |

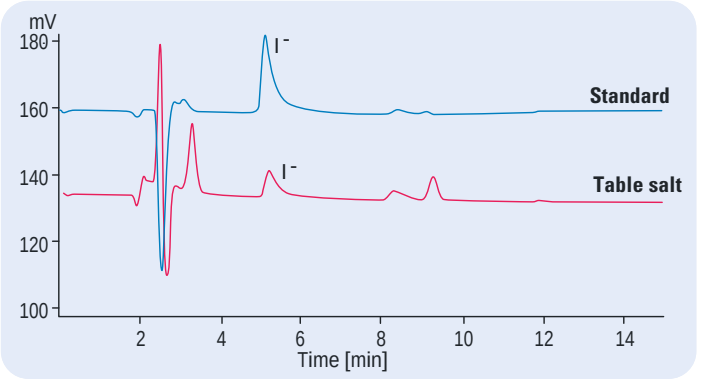
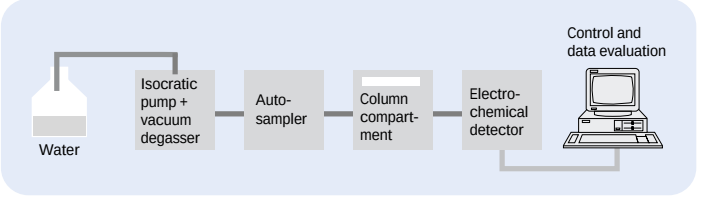



**Figure 24**
**Analysis of iodide in table salt**




17. A.G. Huesgen, R. Schuster, "Analysis of selected anions with HPLC and electrochemical detection", *Agilent Application Note* 5091-1815E, **1991**.

# Lipids

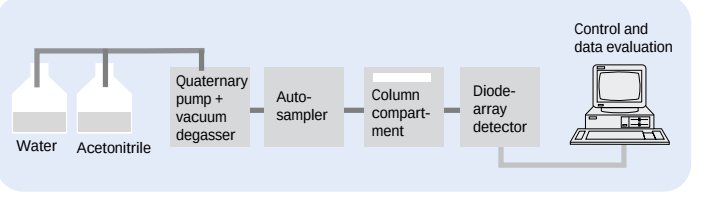
Both saturated and unsaturated triglycerides have been analyzed. Fats and oils are complex mixtures of triglycerides, sterols, and vitamins. The composition of triglycerides is of great interest in food processing and dietary control. Owing to the low stability of triglycerides containing unsaturated fatty acids, reactions with light and oxygen form hydroperoxides, which strongly influence the taste and quality of fats and oils. Adulteration with foreign fats and the use of triglycerides that have been modified by a hardening process also can be detected through triglyceride analysis.

## Triglycerides and hydroperoxides in oils

The HPLC method presented here was used to analyze triglycerides, hydroperoxides, sterols, and vitamins with UV-visible diode-array detection (UV-DAD). Spectra were evaluated in order to trace hydroperoxides and to differentiate saturated from unsaturated triglycerides. Unsaturated triglycerides in olive oil have a very distinctive pattern. Other fats and oils are also complex mixtures of triglycerides but exhibit an entirely different pattern. Adulteration with foreign fats and the use of refined triglycerides in olive oil also can be detected through triglyceride analysis.

### Sample preparation

Triglycerides can be extracted from homogenized samples with petrol ether. Fats and oils can be dissolved in tetrahydrofuran.[17]



| | |
|---|---|
| Sample preparation | Samples were dissolved in tetrahydrofuran (THF). |
| Column | 200 x 2.1 mm Hypersil MOS, 5 µm |
| Mobile phase | A = water<br>B = ACN/methyl-tert.butylether (9:1) |
| Gradient | at 0 min 87 % B<br>at 25 min 100 % B |
| Post time | 4 min |
| Flow rate | 0.8 ml/min |
| Column compartment | 60 °C |
| Injection volume | 1 µl standard |
| UV absorbance | 200 nm and 215 nm to detect triglycerides<br>240 nm to detect hydroperoxides<br>280 nm to detect tocopherols and decomposed triglycerides (fatty acids with three conjugated double bonds) |

**HPLC method performance**

| | |
|---|---|
| Limit of detection | |
| for saturated triglycerides | > 10 µg |
| for unsaturated triglycerides | |
| fatty acids with 1 double bond | >150 ng |
| fatty acids with 2 double bonds | > 25 ng |
| fatty acids with 3 double bonds | < 10 ng |
| Repeatability of | |
| RT over 10 runs | < 0.7 % |
| areas over 10 runs | < 6 % |

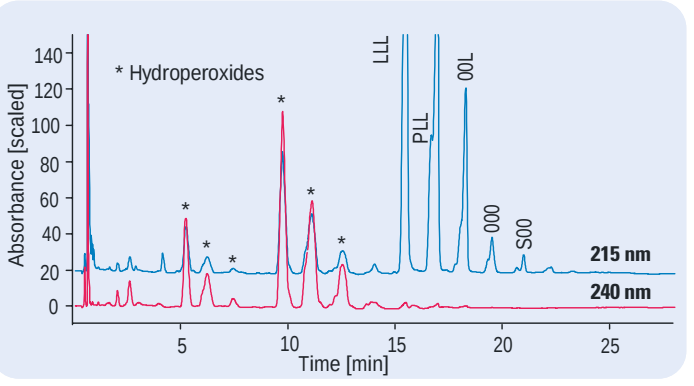



**Figure 25**
**Triglyceride pattern of aged sunflower oil. The increased response at 240 nm indicates hydroperoxides**

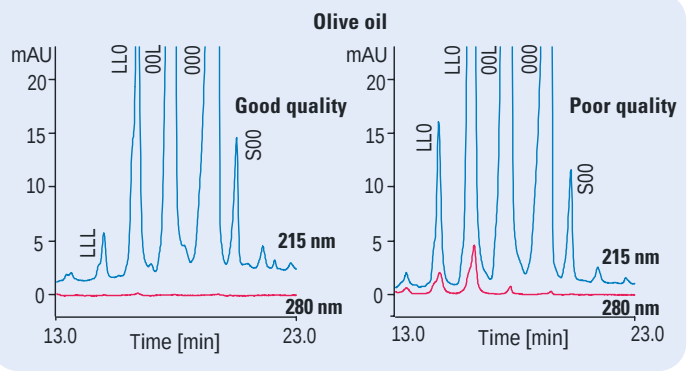



**Figure 26**
**Analysis of olive oil. The response at 280 nm indicates a conjugated double bond and therefore poor oil quality**

## Triglycerides in olive oil

Unsaturated triglycerides in olive oil have very characteristic patterns. Other fats and oils are also complex mixtures of triglycerides but with different patterns.

### Sample preparation information

Triglycerides can be extracted from homogenized samples with petrol ether. Fats and oils can be dissolved in tetrahydrofurane.

### Chromatographic conditions

The presented HPLC method was used to analyze the unsaturated triglycerides, LnLnLn, LLL, and OOO.[18]

| | |
|---|---|
| Sample preparation | Samples were dissolved in tetrahydrofurane. |
| Column | 200 × 2.1 mm Hypersil MOS, 5 µm |
| Mobile phase | acetone/ACN (30:70) |
| Flow rate | 0.5 ml/min |
| Column compartment | 30 °C |
| Injection volume | 2 µl |
| Detector | refractive index |

**HPLC method performance**

| | |
|---|---|
| Limit of detection for ECD | 50 µg/l with S/N = 2 |
| Repeatability of RT over 10 runs | < 0.3 % |
| areas over 10 runs | 5 % |

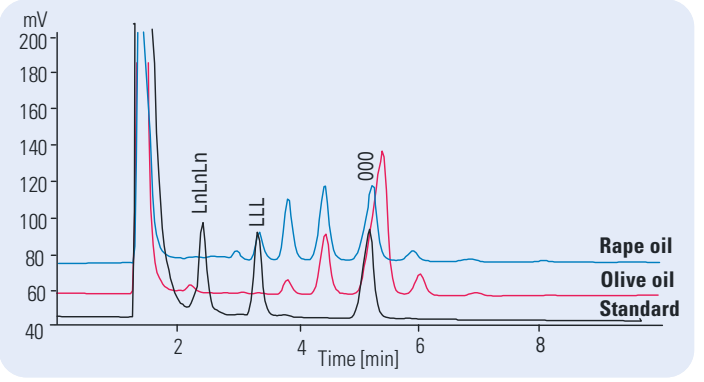
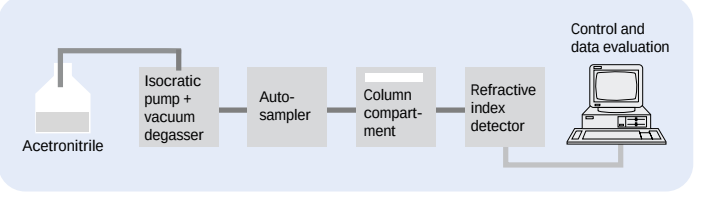



**Figure 27**
**Analysis of the triglyceride pattern of olive and rape oil**




18. "Determination of triglycerides in vegetable oils", EC Regulation No. L248, 28ff.

## Fatty acids

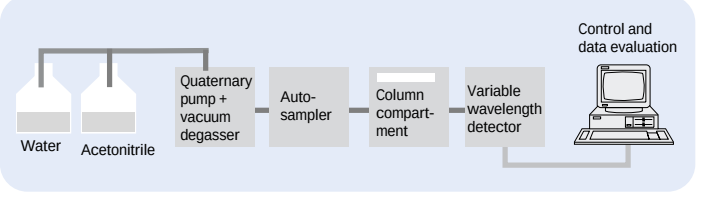
Saturated and unsaturated fatty acids from $C_4$ through $C_{22}$ have been analyzed. Fatty acids are the primary components of oils and fats and form a distinctive pattern in each of these compounds. For example, butter and margarines can be differentiated by the percentage of butyric acid in the triglycerides. To determine the fatty acid pattern of a fat or oil, free fatty acids first are obtained through hydrolysis. Derivatization is then performed to introduce a chromophore, which enables analysis of the fatty acids using HPLC and UV-visible detection.

### Sample preparation

The triglycerides were hydrolyzed using hot methanol and KOH, followed by derivatization.

### Chromatographic conditions

The HPLC method presented here was used in the analysis of the fatty acid pattern of dietary fat. The method involves hydrolysis with hot KOH/methanol and online derivatization with bromophenacyl bromide.



Sample preparation

0.215 g fat was hydrolyzed with 500 µl MEOH/ KOH at 80 °C for 40 min in a thermomixer. After cooling 1.5 ml ACN/THF (1:1) was added, and the mixture was shaken for 5 min. The mixture was then filtered through a 0.45-µm Minisart RNML from Satorius.

| | |
|---|---|
| Column | 200 x 2.1 mm, MOS, 5 µm |
| Mobile phase | A = water (70 %)<br>B = (ACN + 1 % THF) (30 %) |
| Gradient | at 5 min 30 % B<br>at 15 min 70 % B<br>at 17 min 70 % B<br>at 25 min 98 % B |
| Flow rate | 0.3 ml/min |
| Column compartment | 50 °C |
| Detector | variable wavelength, 258 nm |
| Derivatization | 60 mg/ml bromophenacyl bromide was dissolved in ACN. |

Injector program for online derivatization

1. Draw 2.0 µl from vial 2 (ACN)
2. Draw 1.0 µl from air
3. Draw 1.0 µl from vial 3 (derivatization agent)
4. Draw 0.0 µl from vial 4 (wash bottle) (ACN/THF, 50:50)
5. Draw 1.0 µl from sample
6. Draw 0.0 µl from vial 4 (wash bottle)
7. Draw 1.0 µl from vial 3 (derivatization agent)
8. Draw 0.0 µl from vial 4 (wash bottle)
9. Draw 1.0 µl from vial 5 (acetonitrile + 5 % TEA)
10. Draw 0.0 µl from vial 4 (wash bottle)
11. Mix 9 µl in air, 30 µl/min speed, 10 times
12. Wait 2.0 min
13. Inject

**HPLC method performance**

| | |
|---|---|
| Limit of detection | 200 pg injected amount, S/N = 2 |
| Repeatability of | |
| RT over 10 runs | < 0.1 % |
| areas over 10 runs | 5 % |

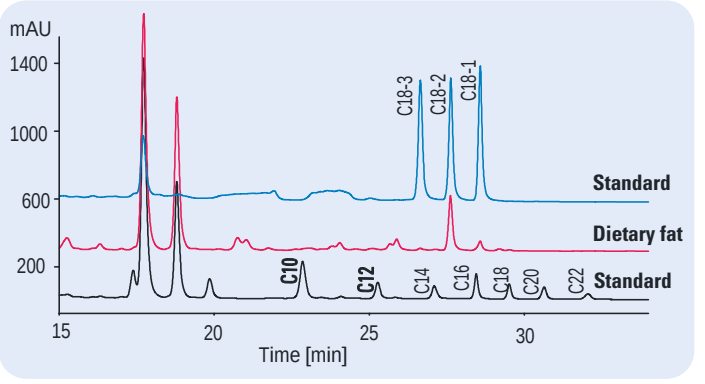



**Figure 28**
**Analysis of a dietary fat triglyceride pattern. Overlay of one sample and two standard chromatograms**

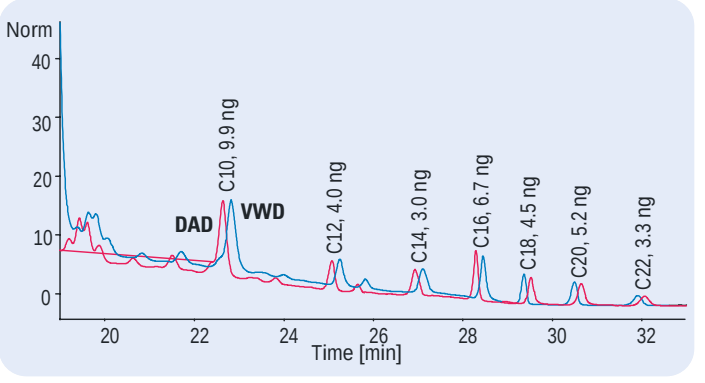


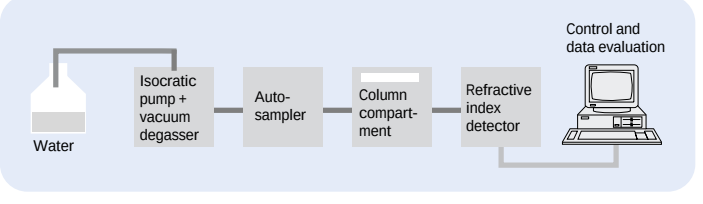

**Figure 29**
**Trace analysis of triglycerides with a diode-array and a variable wavelength detector in series**

## Carbohydrates

The following carbohydrates have been analyzed: glucose, galactose, raffinose, fructose, mannitol, sorbitol, lactose, maltose, cellobiose, and sucrose. Food carbohydrates are characterized by a wide range of chemical reactivity and molecular size. Because carbohydrates do not possess chromophores or fluorophores, they cannot be detected with UV-visible or fluorescence techniques. Nowadays, however, refractive index detection can be used to detect concentrations in the low parts per million (ppm) range and above, whereas electrochemical detection is used in the analysis of sugars in the low parts per billion (ppb) range.

### Sample preparation

Degassed drinks can be injected directly after filtration. More complex samples require more extensive treatment, such as fat extraction and deproteination. Sample cleanup to remove less polar impurities can be done through solid-phase extraction on C18 columns.



## Chromatographic conditions

The HPLC method presented here was used to analyze mono-, di-, and trisaccharides as well as sugar alcohols.

| | |
|---|---|
| Sample preparation | Samples were directly injected. |
| Column | 300 x 7.8 mm Bio-Rad HPXP, 9 µm |
| Mobile phase | water |
| Column compartment | 80 °C |
| Flow rate | 0.7 ml/min |
| Detector | refractive index |

**HPLC method performance**

| | |
|---|---|
| Limit of detection | < 10 ng with S/N = 2 |
| Repeatability of RT over 10 runs | < 0.05 % |
| areas over 10 runs | 2 % |

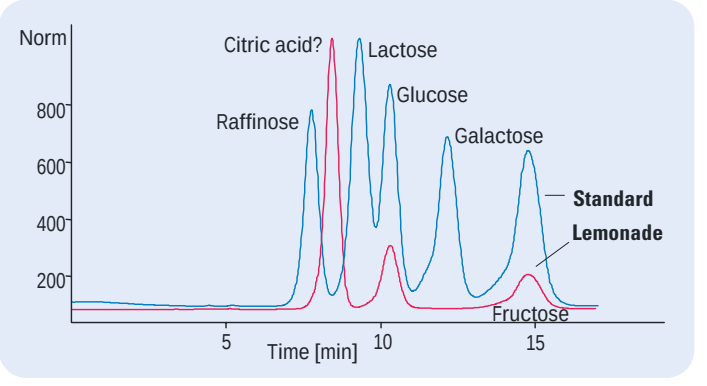



**Figure 30**
**Analysis of carbohydrates in lemonade**

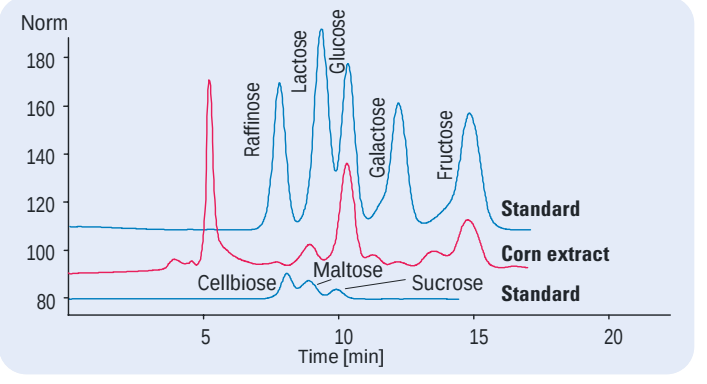



**Figure 31**
**Analysis of carbohydrates in corn extract**


4. *Official Methods of Analysis, Food Compositions; Additives, Natuaral Contaminants*, 15th ed; AOAC: Arlington, VA, **1990**, Vol. 2; AOAC Official Method 980.13: Fructose, glucose, lactose, maltose, sucrose in milk chocolate; AOAC Official Method 982.14: Glucose, fructose, sucrose, and maltose in presweetened cereals; AOAC Official Method 977.20: Separation of sugars in honey; AOAC Official Method 979.23: Saccharides (major) in corn syrup; AOAC Official Method 983.22: Saccharides (minor) in corn syrup; AOAC Official Method 984.14: Sugars in licorice extracts.

# Vitamins

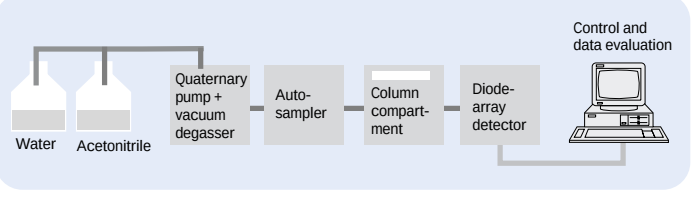
Fat-soluble vitamins, such as vitamins E, D, and A, and water-soluble vitamins, such as vitamins C, $B_6$, $B_2$, $B_1$, and $B_{12}$, have been analyzed.

Vitamins are biologically active compounds that act as controlling agents for an organism's normal health and growth. The level of vitamins in food may be as low as a few micrograms per 100 g. Vitamins often are accompanied by an excess of compounds with similar chemical properties. Thus not only quantification but also identification is mandatory for the detection of vitamins in food. Vitamins generally are labile compounds that should not exposed to high temperatures, light, or oxygen. HPLC separates and detects these compounds at room temperature and blocks oxygen and light.[19] Through the use of spectral information, UV-visible diode-array detection yields qualitative as well as quantitative data. Another highly sensitive and selective HPLC method for detecting vitamins is electrochemical detection.

## Water-soluble vitamins

### Sample preparation

Different food matrices require different extraction procedures.[19] For simple matrices, such as vitamin tablets, water-soluble vitamins can be extracted with water in an ultrasonic bath after homogenization of the food sample.



## Chromatographic conditions for UV detection

The HPLC method presented here was used to analysis vitamins in a vitamin drink.

| | |
|---|---|
| Sample preparation | filtration |
| Column | 100 x 4 mm<br>Hypersil BDS, 3 µm |
| Mobile phase | A= water with pH = 2.1 ($H_2SO_4$) = 99 %<br>B = ACN 1 % |
| Gradient | at 3.5 min 1 % B<br>at 11 min 25 % B<br>at 19 min 90 % B |
| Post time | 6 min |
| Flow rate | 0.5 ml/min |
| Column compartment | 30 °C |
| Injection volume | 2–5 µl |
| Detector | UV-DAD<br>detection wavelength 220/30 nm,<br>reference wavelength 400/100 nm |

**HPLC method performance**

| | |
|---|---|
| Limit of detection | < 500 pg (injected amount), S/N = 2 |
| Repeatability of | |
| RT over 10 runs | < 0.2 % |
| areas over 10 runs | < 2 % |

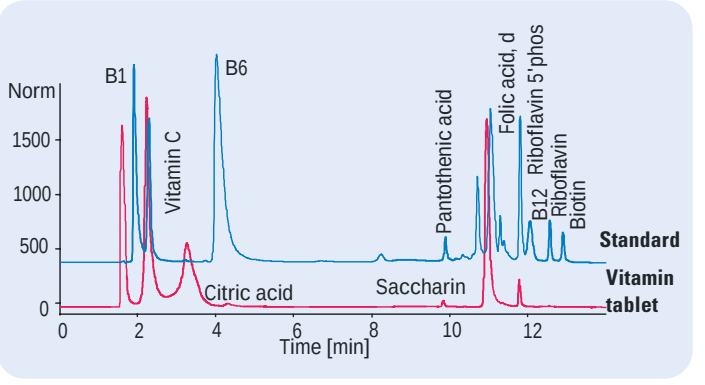



**Figure 32**
**Analysis of water-soluble vitamins in a vitamin tablet**

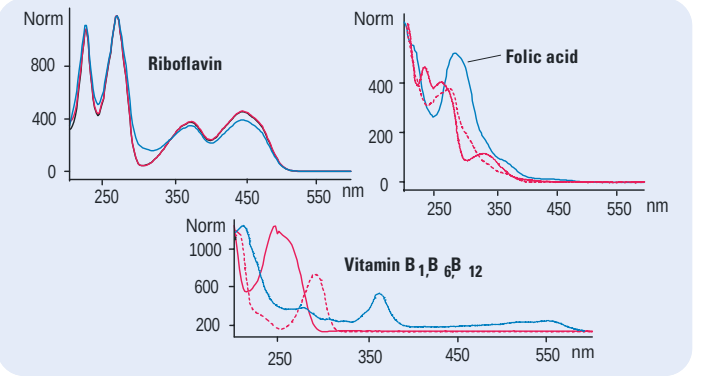



**Figure 33**
**Spectra of water-soluble vitamins**

19. L.M. Nollet, *" Food Analysis by HPLC"*, New York, **1992**.

## Chromatographic conditions for electrochemical detection

The HPLC method presented here was used in the analysis of vitamins in animal feed.[20]

| | |
|---|---|
| Sample preparation | Vitamin preparation was diluted with water 1:100 |
| Column | 125 x 4 mm, Lichrospher RP 18, 5 µm |
| Mobile phase | water + 0.02 M $KH_2PO_4$ + 0.03 M tetrabutylammoniumhydrogensulfat + 0.03 M heptanesulfonic acid + 2 % ACN |
| Stop time | 15 min |
| Flow rate | 0.8 ml/min |
| Column compartment | 30 °C |
| Injection volume | 1 µl standard<br>0.5 µl sample |
| Detector | electrochemical |
| Working electrode: | glassy carbon |
| Operation mode: | amperometry |
| Working potential: | 1.2 V |
| Range: | 0.5 µA |
| Reference electrode: | AgCl/KCl |
| Response time: | 1 s |

**HPLC method performance**

| | |
|---|---|
| Limit of detection | 30 pg (injected amount) S/N = 2 |
| Repeatability of | |
| RT over 10 runs | < 0.5 % |
| areas over 10 runs | < 5 % |
| Linearity | 30 pg to 1 ng |

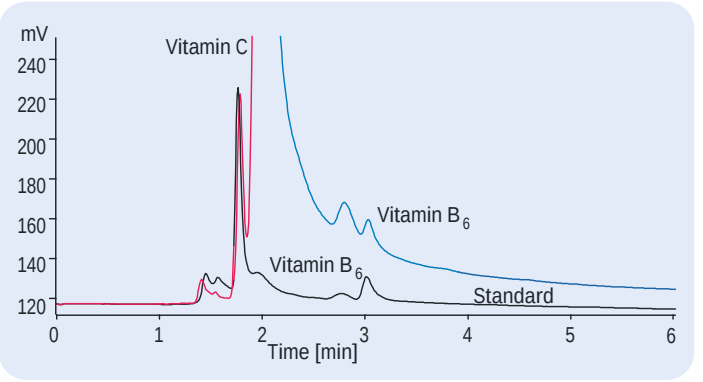
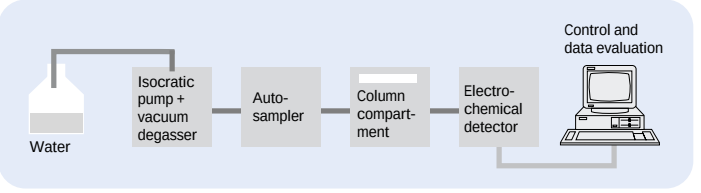



**Figure 34**
**Analysis of vitamin $B_6$ in a vitamin preparation**




20. A.G. Huesgen, R. Schuster, "Analysis of selected vitamins with HPLC and electrochemical detection", *Agilent Application Note 5091-3194E* , **1992**.

## Fat-soluble vitamins

### Sample preparation

Different food matrices require different extraction procedures. These procedures include alkaline hydrolysis, enzymatic hydrolysis, alcoholysis, direct solvent extraction, and supercritical fluid extraction of the total lipid content.

### Chromatographic conditions for UV detection

The HPLC method presented here was used in the analysis of a vitamin standard.

| | |
|---|---|
| Column | 100 x 2.1 mm<br>Hypersil MOS, 5 µm |
| Mobile phase | A = water<br>B = ACN (70 %) |
| Gradient | at 15 min 90 % B<br>at 16 min 95 % B |
| Post time | 3 min |
| Flow rate | 0.5 ml/min |
| Column compartment | 40 °C |
| Injection volume | 2–5 µl |
| Detector | UV-DAD<br>detection wavelengths<br>230/30 nm, 400/100 nm;<br>reference wavelengths<br>280/40 nm, 550/100 nm |

**HPLC method performance**

| | |
|---|---|
| Limit of detection | 1 ppb with S/N = 2 |
| Repeatability of | |
| RT over 10 runs | < 0.82 % |
| areas over 10 runs | < 2.2 % |

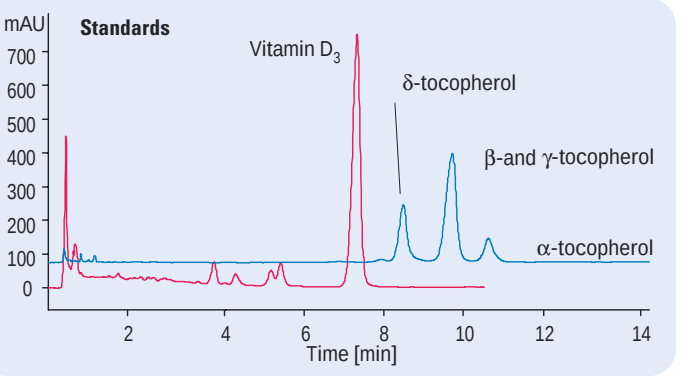
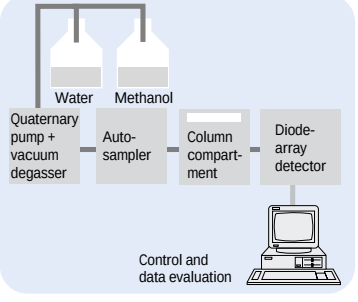



**Figure 35**
**Analysis of fat-soluble vitamins with UV detection**



### Chromatographic conditions for electrochemical detection

The HPLC method presented here was used in the analysis of a vitamin standard.[20]

| | |
|---|---|
| Column | 125 x 4 mm Lichrospher RP18, 5 µm |
| Mobile phase | methanol + 5 g/l lithiumperchlorate + 1 g/l acetic acid |
| Stop time | 20 min |
| Flow rate | 1 ml/min |
| Oven temperature | 30 °C |
| Injection volume | 1 µl standard |
| Detector | electrochemical |
| Working electrode: | glassy carbon |
| Operation mode: | amperometry |
| Working potential: | 0.9 V |
| Range: | 0.5 µA |
| Reference electrode: | AgCl/KCl |
| Response time: | 8 s |

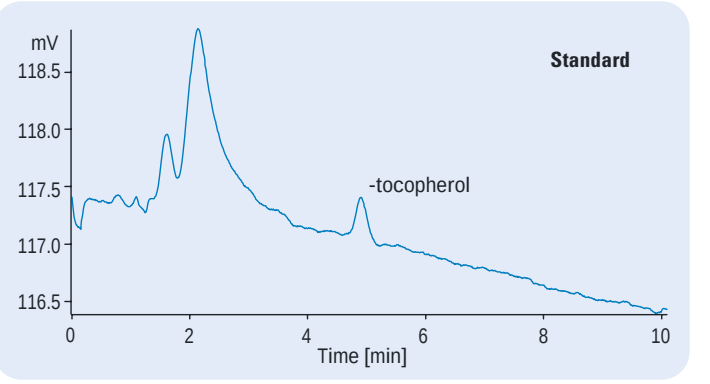
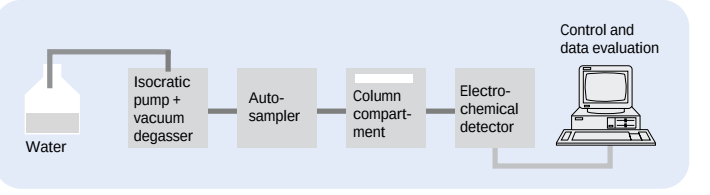



**Figure 36**
**Analysis of a fat-soluble vitamin with electrochemical detection**

**HPLC method performance**

| | |
|---|---|
| Limit of detection | 80 pg (injected amount), S/N = 2 |
| Repeatability of | |
| RT over 10 runs | < 0.5 % |
| areas over 10 runs | < 5 % |
| Linearity | 30 pg to 1 ng |




## Analysis of tocopherols on normal-phase column

Tocopherols cannot be separated completely using reversed-phase chromatography. However, normal-phase chromatography can separate isocratically all eight tocopherols (T) and tocotrienols ($T_3$ ) naturally occurring in fats, oils, and other foodstuffs. Fluorescence detection is recommended for the analysis of total lipid extraction because UV absorbance detection is not selective enough to prevent detection of coeluting peaks.

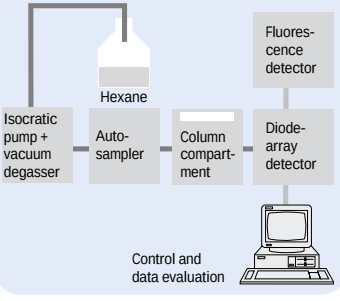
## Chromatographic conditions for analysis of tocopherols on normal-phase column

The HPLC method presented here was used in the analysis of margarine.

| | |
|---|---|
| Sample preparation | 20 g sample dissolved in 15 ml hexane |
| Column | 100 x 2.1 mm Hypersil SI 100, 5 µm |
| Mobile phase | hexane + 2 % isopropanol |
| Stop time | 8 min |
| Flow rate | 0.3 ml/min |
| Column compartment | 25 °C |
| Injection volume | 0.5 µl |
| Detector | |
| UV-DAD | 295/80 nm |
| Fluorescence | excitation wavelength 295 nm, emission wavelength 330 nm |

**HPLC method performance**

| | |
|---|---|
| Limit of detection for diode-array | 10–20 ng, S/N = 2 |
| Limit of detection for fluorescence | 0.5–2 ng S/N = 2 |
| Repeatability of RT over 10 runs | < 2 % |
| areas over 10 runs | < 2 % |



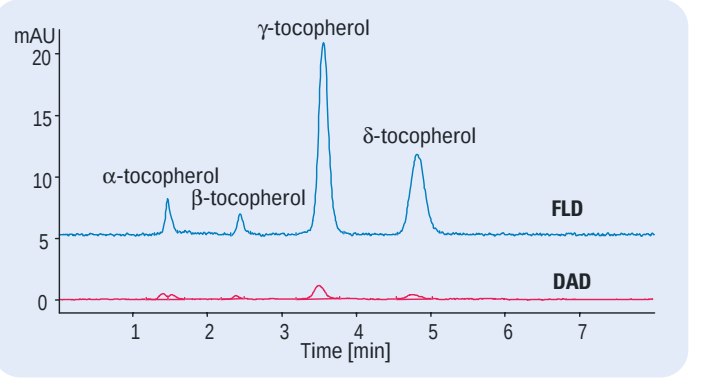




**Figure 37**
**Analysis of tocopherols on normal phase using UV and fluorescence detection**

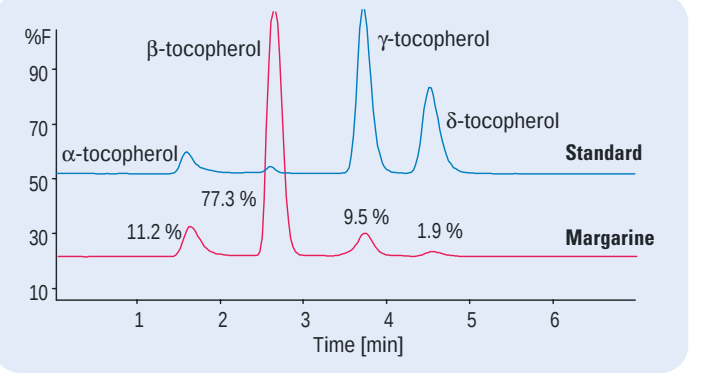



**Figure 38**
**Analysis of tocopherol concentration in margarine fat extract with fluorescence detection**

## Biogenic amines

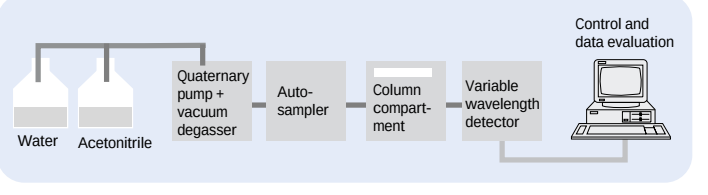
The following amines were analyzed: ammonia, amylamine, 1-butylamine, 1,4-diaminobutane, 1,5-diaminopentane, diethylamine, ethanolamine, ethylamine, hexylamine, histamine, isobutylamine, isopropylamine, methylamine, 3-methylbutylamine, morpholine, phenethylamine, propylamine, pyrrolidine, and tryptamine.

Free amines are present in various food products and beverages, including fish, cheese, wine, and beer.

High concentrations of specific amines can have toxic properties. As a result, several countries have set maximum tolerance levels for these compounds in foodstuffs. HPLC is now preferred for the analysis of amines in food matrices because of its shorter analysis time and relatively simple sample preparation.

### Sample preparation

Amines can be extracted from different matrixes using liquid/liquid extraction or solid-phase extraction followed by derivatization.



## Chromatographic conditions for UV detection

The HPLC method presented here was used to analyze amines in wine.[21]

Sample preparation
25 ml wine was decolored with polyvinylpyrrolidoine. After filtration, the amines (5 ml sample, pH = 10.5) were derivatized with 2 ml dansyl chloride solution (1 %). The reaction solution was cleaned with solid-phase extraction using C18 cartridges (500 mg). After elution with 2 ml ACN, the solution was concentrated to 100 µl.

| | |
|---|---|
| Column | 250 x 4.6 mm Spherisorb ODS2, 5 µm |
| Mobile phase | A = water + 5 % ACN = 75 %<br>B = ACN (25 %) |
| Gradient | at 5 min 45 % B<br>at 30 min 45 % B<br>at 50 min 60 % B<br>at 55 min 80 % B<br>at 60 min 80 % B |
| Stop time | 60 min |
| Post time | 4 min |
| Flow rate | 1 ml/min |
| Column compartment | 60 °C |
| Detector | UV-VWD<br>250 nm |

**HPLC method performance**

| | |
|---|---|
| Recovery rate | > 85 % |
| Limit of detection | 50–150 µg/l |
| Method repeatability for 5 red wine analyses | < 5 % |
| Linearity 500 µg/l to | 20 mg/l |

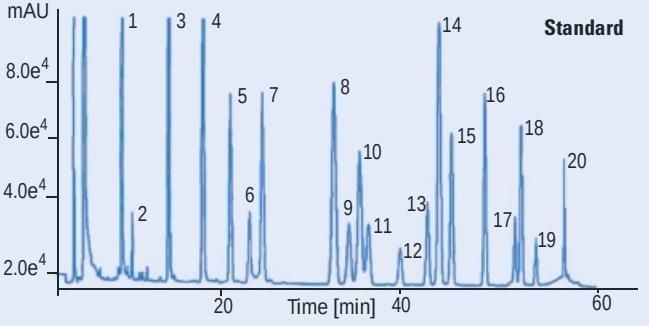



**Figure 39**
**Analysis of amine standard with UV detection after derivatization**

21. O. Busto, et al., "Solid phase extraction applied to the determination of biogenic amines in wines by HPLC", *Chromatographia*, **1994**, 38(9/10), 571–578.

## Amino acids

Both primary and secondary amino acids were analyzed in one run.

The amino acid composition of proteins can be used to determine the origin of meat products and thus to detect adulteration of foodstuffs. Detection of potentially toxic amino acids is also possible through such analysis. Through the use of chiral stationary phases as column material, D and L forms of amino acids can be separated and quantified.

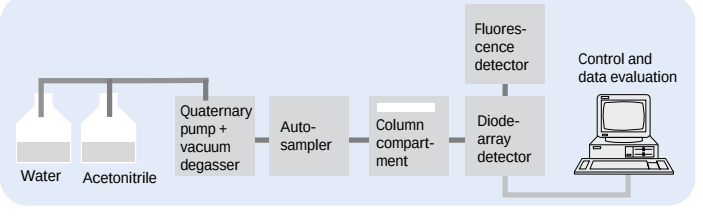
HPLC in combination with automated online derivatization is now a well-accepted method for detecting amino acids owing to its short analysis time and relatively simple sample preparation.

### Sample preparation

Hydrolyzation with HCl or enzymatic hydrolysis is used to break protein bonds.

### Chromatographic conditions

The HPLC method presented here was used in the analysis of secondary and primary amino acids in beer with precolumn derivatization and fluorescence detection.[22, 23]



| | |
|---|---|
| Sample preparation | filtration |
| Column | 200 x 2.1 mm<br>Hypersil ODS, 5 µm |
| Mobile phase | A = 0.03 M sodium acetate<br>pH = 7.2 + 0.5% THF<br>B = 0.1 M sodium acetate/<br>ACN (1:4) |

Gradient
- at 0 min 0 % B at 0.45 ml/min flow rate
- at 9 min 30 % B
- at 11 min 50 % B at 0.8 ml/min flow rate
- at 13 min 50 % B
- at 14 min 100 % B at 0.45 ml/min flow rate
- at 14.1 min at 0.45 ml/min flow rate
- at 14.2 min at 0.8 ml/min flow rate
- at 17.9 min at 0.8 ml/min flow rate
- at 18 .0 min at 0.45ml/min flow rate
- at 18 min 100 % B
- at 19 min 0 % B

| | |
|---|---|
| Post time | 4 min |
| Flow rate | 0.45 ml/min |
| Column compartment | 40 ºC |
| Injection volume | 1 µl standard |

Detector
- UV -DAD 338 nm and 266 nm
- Fluorescence
- Excitation wavelength: 230 nm
- Emission wavelength: 450 nm
- at 11.5 min
- Excitation wavelength: 266 nm
- Emission wavelength: 310 nm
- Photomultiplier gain: 12
- Response time: 4 s

**HPLC method performance**

| | |
|---|---|
| Limit of detection | DAD < 5 pmol<br>FLD < 100 fmol |
| Repeatability of | |
| RT over 6 runs | < 1 % |
| areas over 6 runs | < 5 % |
| Linearity DAD | 1 pmol to 4 nmol |

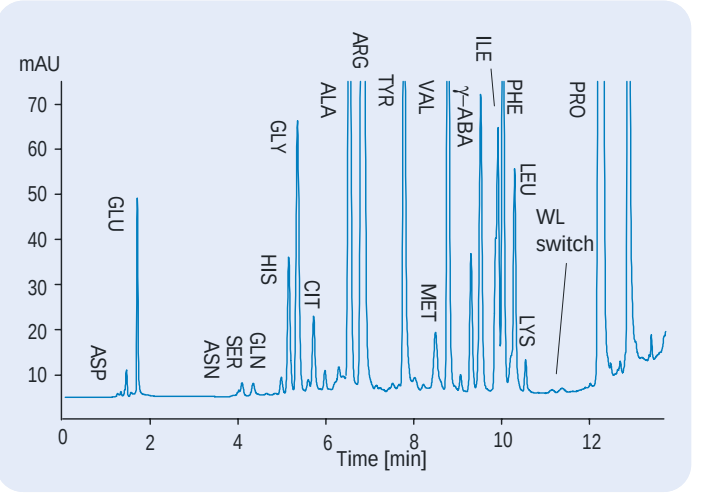



**Figure 40**
**Analysis of amino acids in beer after online derivatization**

Injector program for online derivatization
1. Draw 3.0 µl from vial 2 (borate buffer)
2. Draw 1.0 µl from vial 0 (OPA reagent)
3. Draw 0.0 µl from vial 100 (water)
4. Draw 1.0 µl from sample
5. Draw 0.0 µl from vial 100 (water)
6. Mix 7.0 µl (6 cycles)
7. Draw 1.0 from vial 1 FMOC reagent
8. Draw 0.0 µl from vial 100 (water)
9. Mix 8.0 µl (3 cycles)
10. Inject


22. "Sensitive and reliable amino acid analysis in protein hydrolysates using the Agilent 1100 Series", *Agilent Technical Note* 5968-5658E, **1999**
23. R. Schuster, "Determination of amino acids in biological, pharmaceutical, plant and food samples by automated precolumn derivatization and HPLC", *J. Chromatogr.*, **1988**, 431, 271–284.

# Peptides

### Peptide mapping of phytochrome from dark grown oat seedlings using capillary liquid chromatography

The analyzed phytochrome is a photoreceptor protein that controls light-dependent morphogenesis in plants. For example, potato clod forms pale long sprouts if it germinates in a dark cellar. However, if this process takes place in the light, a normal plant with green leaves grows and photosynthesis occurs. Phytochrome proteins are present in very low concentrations in potato clod, and sample volume and concentration of these proteins is rather low following sample preparation. In this case, columns or capillaries with a small internal diameter are preferred because sensitivity increases with decreasing internal diameter of the column. The use of capillaries with an internal diameter of 100–300 µm enables flow rates as low as 0.5–4.0 µl/min, which reduces solvent consumption. Such flow rates are well-suited to liquid chromatography-mass spectroscopy (LC/MS) electrospray ionization.

In our experience, the appropriate conversion of standard HPLC equipment to a capillary HPLC system is cost-effective and yields the highest performance for running capillary columns. For conversion, a flow stream-split device, a 35-nL capillary flow cell for the detector, and capillary connections between system modules are required. System delay volume should be as low as possible. To meet the demands of such a system, the Agilent 1100 Series binary pump, which has inherently low delay volume, was selected as a pumping system. The flow splitter, the capillary flow cell for the detector, and the column were purchased from LC Packings in Amsterdam.[24]

With this design, a standard flow rate (for example, 100 or 50 µl/min) can be set for the pump. This flow then can be reduced by calibrated splitters between 0.5 and 4 µl/min, for example. This flow rate is optimal for capillary columns with an internal diameter of 300 µm.

## Chromatographic conditions

Capillary HPLC with UV and MS detection has been used in the analysis of phytochrome protein from dark grown oat seedlings. Figures 41, 42 and 43 show the UV and total ion chromatogram together with two mass spectra of selected fragments. The Agilent 1100 Series LC system was used without mixer. All tubings were as short as possible, with an internal diameter of 75–120 µm id.

## Sample preparation

The extracted protein was reduced and alkylated prior to digestion with trypsin.

| | |
|---|---|
| Sample | tryptic digest of phytochrome from oat seedlings, 7 pmol/µl |
| Capillary column | 300 µm x 25 cm, C18 |
| Mobile phase | A = 0.025 % TFA in water<br>B = 0.02 % TFA in ACN |
| Gradient | 0.35 % B/min |
| Flow rate | 100 µl/min split to 4 µl/min |
| Column compartment | 25 °C |
| Injection volume | 2.5 µl |
| Detector | UV-VWD wavelength 206 nm with a 35-nl, 8-mm flow cell |

**HPLC method performance**

| | |
|---|---|
| Limit of detection | 1 pmol |
| Repeatability of | |
| RT over 10 runs | < 0.7 % |
| areas over 6 runs | < 1 % |

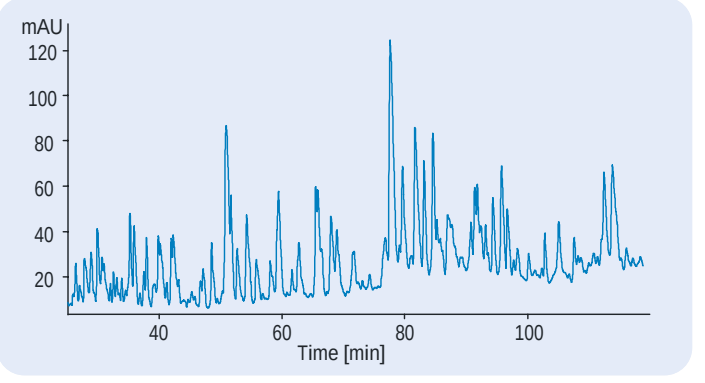
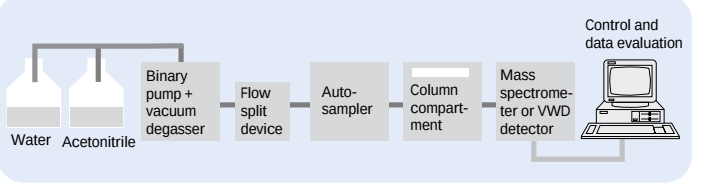



**Figure 41**
**Capillary LC-MS of a phytochrome tryptic digest (17.5 pmol)—UV trace**



MS data was used for further evaluation. Some of the tryptic mass fragments of the phytochrome are signed. As an example, figure 42 shows two mass spectra.

| | |
|---|---|
| Voltages | Vcyl -5500, Vend -3500, Vcap -4000, CapEx 150 |
| Scan | 400–1800 m/z |
| Threshold | 150 |
| Sampling | 1 |
| Stepsize | 0.15 amu |
| Drying gas | nitrogen, 150 °C |
| Nebulizer | gas nitrogen, < 20 psi |

The Agilent 5989B MS engine was equipped with an Iris™ Hexapole Ion Guide

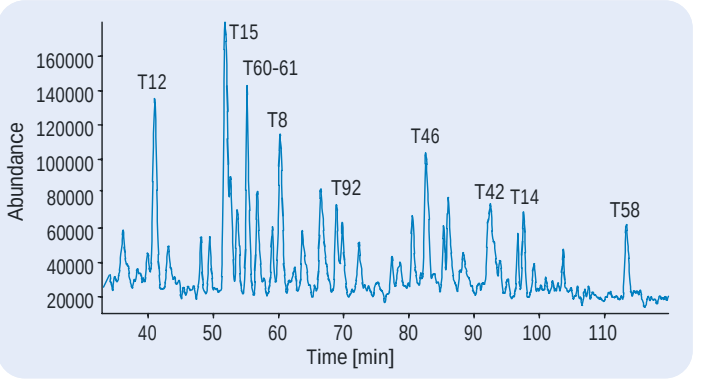



**Figure 42**
**Capillary LC-MS of a phytochrome tryptic digest (17.5 pmol)—total ion chromatography (TIC)**

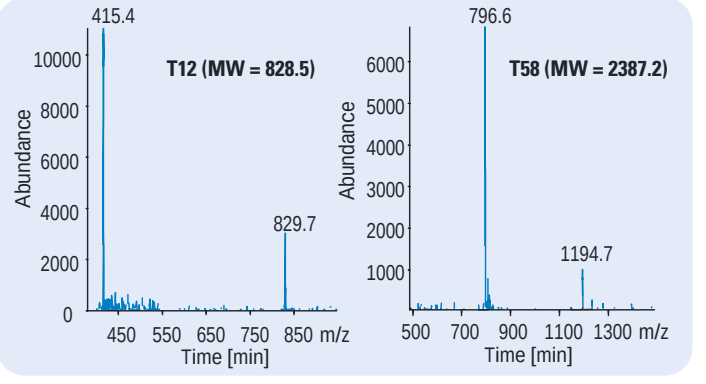


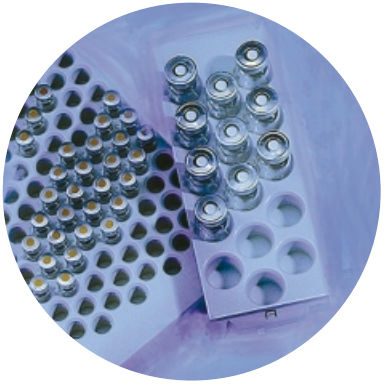

**Figure 43**
**Mass spectra of T12 and T58**

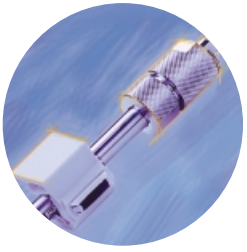
24. “Capillary Liquid Chromatography with the Agilent 1100 Series Modules and Systems for HPLC”, *Agilent Technical Note 5965-1351E* , **1996**.

# The Equipment Basics

# Part Two

An overview of the hardware and the software components needed for successful HPLC, and an introduction to the analytical techniques that have become routine in food analysis





*Chapter 4*

# Separation in the liquid phase

**Liquid chromatography offers a wide variety of separation modes and mobile phases for optimizing your separation system.**

## Separation mechanisms

Stationary phases can be classified according to the mechanism by which they separate molecules:

- partition phases
- adsorption phases
- ion-exchange phases
- size-exclusion phases

Nowadays the most popular column material is reversed phase, in which separation is achieved through partition and through adsorption by unprotected silanol groups. In reversed-phase chromatography, the stationary phase is nonpolar (or less polar than in the mobile phase) and the analytes are retained until eluted with a sufficiently polar solvent or solvent mixture (in the case of a mobile-phase gradient).

### Reversed-phase materials

Reversed-phase materials have wide application and a long lifetime. Moreover, these media have good batch-to-batch reproducibility, low equilibration times, high mechanical stability, and predictable elution times and elution order. Reversed-phase chromatography is frequently used in food analysis, as shown in part one of this primer.

### Ion-exchange materials

Compared with reversed-phase media, ion-exchange materials have a shorter lifetime, are less mechanically stable, and take longer to equilibrate. These columns have limited application in food analysis and are used primarily for inorganic cations and anions or for glyphosate.

**Size-exclusion gels**

Size-exclusion chromatography is used for sample cleanup and fractionation and is described in more detail in chapter 5 (“Sample preparation”).

**Adsorption media**

Adsorption chromatography is used for sampling and cleanup. For example, flavonoids from plant material can be cleaned, fractionated, and enriched on alumina. Other examples are given in chapter 5.

## The advent of narrow-bore columns

Discussions of HPLC methods often revolve around the internal diameter (id), or bore, of the column to be used. Standard-bore columns have an internal diameter of approximately 4 or 5 mm, whereas narrow-bore columns have an internal diameter of approximately 2 mm. When packed with the same materials as the standard-bore column, the narrow-bore column can achieve the same resolving power with less solvent because the analytes can be eluted at a lower flow rate (< 0.5 ml/min) than the 2–3 ml/min required for standard-bore columns. In addition, narrow-bore columns are 4–6 times more sensitive using the injection volume required for a standard-bore column (see figure 44).

Narrow-bore columns nonetheless place higher demands on the equipment used than standard-bore columns. First, the HPLC pump must yield these low flow rates in a way that is both reproducible and precise. Second, all capillary connections, that is, from injector to column and from column to detector, must be kept to a minimum. Third, because column frits block more often, guard columns are recommended. An HPLC system designed for narrow-bore columns (low dead volume and high-performance pumping system) can achieve solvent economies of more than 60 % as well as improve detection limits with the same injection volume. Moreover, under the same conditions, a standard-bore column may have higher resolving power.

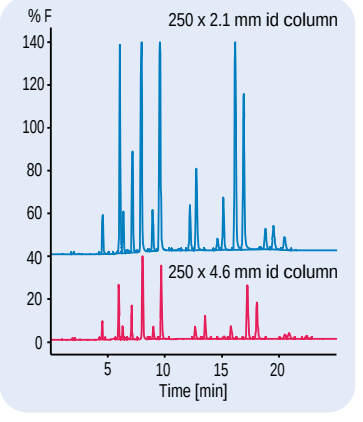



**Figure 44**
**Effect of bore dimensions on separation**

### Influence of column temperature on separation

Many separations depend not only on the column material and mobile phases but also on the column temperature. In such cases, column temperature stability is the dominating factor for the elution order. A thermostatted column compartment using Peltier control with good ambient temperature rejection ensures stable chromatographic conditions. Periodic fluctuations in room temperature during 24-hour use influence these conditions. Figure 45 shows the advantage of Peltier control over conventional air cooling.

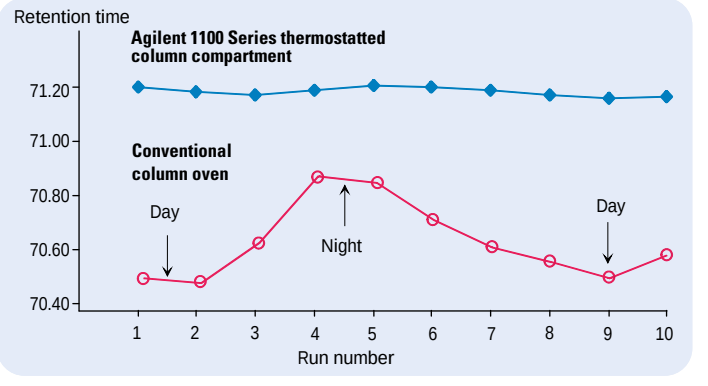
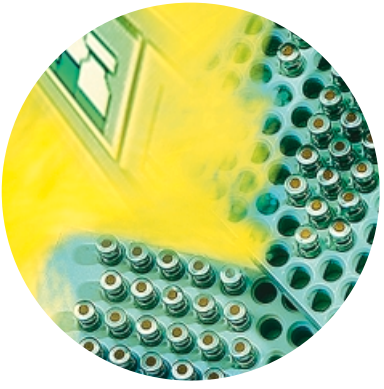



**Figure 45**
**Comparison of Peltier and conventional cooling as demonstrated using retention time fluctuations of a peptide peak over a sequence of 10 consecutive tryptic peptide maps**

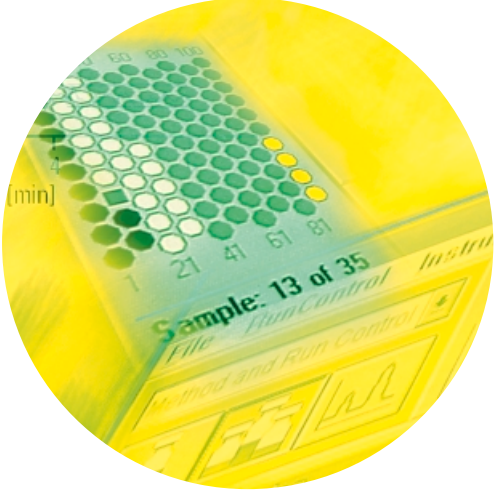
## In brief…

Reversed-phase stationary phases are the most popular LC media for the resolution of food mixtures. The use of narrow-bore columns can result in gains in sensitivity and reduced solvent consumption. For example, these columns have been applied successfully in the analysis of aflatoxins and fatty acids.



*Chapter 5*

# Sample preparation



**The isolation of analytes from other matrix constituents is often a prerequisite for successful food analysis. The broad selection of cleanup and enrichment techniques takes into account the many matrices and compound classes under study.**

## Sample preparation steps

Sample preparation for HPLC can be broken down into the following main steps:

1. **Sampling**
   Collection
   Storage
2. **Cleanup/enrichment offline**
   Homogenization, centrifugation, precipitation, hydrolyzation, liquid/liquid extraction, solid-phase extraction, ultrasonic bath liquid extraction, supercritical fluid extraction, concentration
3. **Cleanup/enrichment online**
   Guard columns
   Online solid-phase extraction
   Gel-permeation chromatography (GPC)
4. **Chemical derivatization**
   Precolumn, online, or offline
   (see also discussion of postcolumn derivatization, chapter 9)

### Automation

Manual extraction, cleaning, and concentration of the sample prior to transfer to the HPLC instrument is time-consuming and can drain resources. Sample preparation therefore should be automated where possible. Nowadays the sample can be fractionated and/or derivatized automatically.

Supercritical fluid extraction (SFE) systems and automated solid-phase extraction equipment also have been interfaced directly to liquid chromatographs. Equipment used to automate preparation of HPLC samples includes:

- Valves—Valves are used to switch to guard columns and online solid-phase extraction techniques.[25] Switching valves are common in HPLC, and some instruments even have built-in column compartment valves. With a six-port valve, for example, the eluant stream can be switched from one column to another to cut out a peak. This peak is then analyzed on the second column.
- SFE interfaces—This technique is rather new, and online systems are under development.[26] An offline procedure has been used successfully in the analysis of vitamin K in infant formula.[27]
- Precolumn derivatization—This well-accepted and commercially available technique[28] has been applied in the analysis of amino acids in beer (see page 50 ff.). Reagents also can be used postcolumn (see page 28).
- Automated solid-phase extraction—This relatively new technique is used to analyze bittering compounds in beer.[29]

## Solids

Solid samples, for example chocolate or meat, should be homogenized before such techniques as steam distillation, SFE, or ultrasonic-stimulated liquid extraction are applied.[30]

### Ultrasonic bath liquid extraction

Ultrasonic bath liquid extraction is a very simple extraction method. Selectivity is achieved through the use of appropriate solvents. Antioxidants and preservatives can be extracted with this technique if the matrix is low in fat.

| ✔ | ✘ |
|---|---|
| Uses relatively small quantities of organic solvents, thereby reducing costs and facilitating disposal. Extraction times are in minutes rather than hours. | Samples high in fat cannot be extracted. |

### Steam distillation

Steam distillation is only used to extract volatile compounds from solid homogenized matrices. For example, biphenyl and 2-phenylphenol pesticides can be extracted from citrus fruits with this technique.[31]

| ✔ | ✘ |
|---|---|
| Enables selective extraction of volatile compounds. | Extraction times are long and offline. Narrow range of use. |

### Supercritical fluid extraction

Until now, supercritical fluid extraction (SFE) was rarely used in food analysis. However, the input of modern SFE instruments can be automated with sampling devices. This method is used primarily for GC,[26, 32] although LC coupling also has been performed with SFE.[33]

| ✔ | ✘ |
|---|---|
| Uses small quantities of organic solvents, thereby reducing costs and facilitating disposal. Extraction times are in minutes rather than hours. Can be automated. | Weak solvating power limits range of analytes. Ultrapure fluids for trace analysis are not always available. |

## Liquids

Liquid-liquid extraction, on- and offline solid-phase extraction, and GPC are used in the analysis of liquid samples or extracts from solid samples.

### Liquid-liquid extraction

Liquid-liquid extraction is the most common extraction method. It requires an appropriate solvent and a separating funnel, or a continuous or counter-current distribution apparatus

| ✔ | ✘ |
|---|---|
| Simple, with highly selective modifiers (pH, salts, or ion-pairing reagents). | Requires large amounts of toxic solvents, can emulsify, and is difficult to automate. |

### Solid-phase extraction

Suitable for cleaning clear liquids such as filtered beverages, solid-phase extraction (SFE) is simple in principle. The sample is first sucked through a preconditioned cartridge or disk filled with adsorbents. The solid then traps the compounds of interest, which can be extracted later with a small amount of an organic eluant. A variety of materials provides a choice of selectivities for use as a fractioning tool. Two or more separate cartridges filled with specific adsorption materials can trap individual fractions of the sample.

SPE is one of the fastest-growing sample preparation and cleanup techniques.[34] Attempts have been made to automate both the procedure and its interface with the chromatograph. Systems based on robotics and valves are available. Pumping a certain volume of water sample through one or more precolumns filled with extraction materials will extract and concentrate the compounds of interest. After desorption with a suitable solvent, the analytes can be introduced into a liquid or gas chromatograph for identification and quantification. The precolumns are

exchanged automatically between analyses to prevent clogging and memory effects.[35] So far this system has been used only to extract pesticides and polynuclear aromatics in river water. A different online solid-phase extraction system has been used to extract and analyze iso-a-acids in beer.[36, 40]

| ✔ | ✘ |
|---|---|
| Uses small amounts of organic solvents, can run several samples at once, and can be automated. | Differing batch-to-batch efficiencies can reduce reproducibility. Risk of irreversible adsorption. Degradation by surface catalysis can occur. |

### Gel permeation chromatography

Also known as size-exclusion chromatography, gel permeation chromatography (GPC) has become a standard technique for isolating compounds of low molecular weight from samples that contain compounds of high molecular weight, such as oil or fats. The separation is based on differences in size, with higher molecular weight compounds retained less than smaller ones. GPC has been used successfully to separate vitamins A, D, and E from glycerides in infant formula and clean-up of pesticides in spices (see chapter 2, page 22 ff).[37]

| ✔ | ✘ |
|---|---|
| Highly reproducible, good automation possibilities. | Large amounts of solvents needed, separation efficiency may differ from batch to batch. |

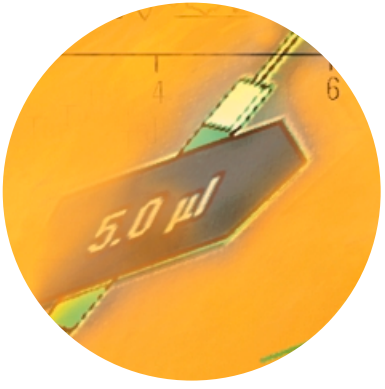
### Guard columns

A guard column is connected in front of the analytical column to prevent contamination of the analytical column by the matrix. Either the guard column can be included in analytical column design, or both columns can be interconnected by a valve that, when switched, transfers fractions from the precolumn to the analytical column. The latter technique is more flexible and can be used for sample cleanup and enrichment. Alternatively, a backflush valve can be used to enrich the sample on a precolumn. Reversing the direction of flow transfers compounds concentrated from the precolumn to the analytical column.

| ✔ | ✘ |
|---|---|
| Highly reproducible, good automation possibilities. | More complex, and more expensive if a valve is used. |

## In brief…

Many food analyses are governed by officially recognized methods, which often include details on sample preparation. Recent trends toward automated sample preparation increase precision by eliminating operator variances. Should you adopt a newly developed sample preparation technique, however, please be aware that the method must comply with existing good laboratory practice (GLP) regulations and with accreditation standards.[38]



# *Chapter 6*

## Injection techniques

**After the sample has been prepared for introduction onto the LC column, analysis can begin. Judgements based on analyte concentration require a reliable quantity of sample volume. The process of introducing the sample onto the column with precision syringes can be automated for increased throughput.[39, 40]**

## Characteristics of a good sample introduction device

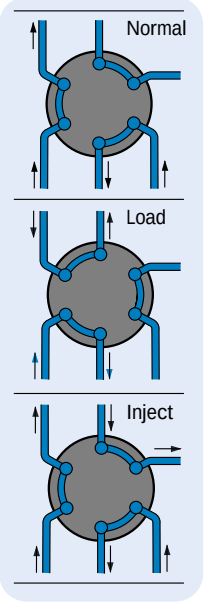



**Figure 46**
**A typical 6-port injection valve**

The main requirements for any sampling device are good precision of injection volumes, low memory effects (carry-over of material from one injection to another), and the ability to draw viscous samples and inject variable volumes. Modern sampling systems can further increase productivity with features such as online precolumn derivatization for selective detection, heating and cooling for improved stability, and microsampling of material in low supply. Some analyses may require corrosive solvents or mobile-phase additives such as 0.1 N HCl or 60 % formic acid. Some vendors supply devices of corrosion-resistant titanium to solve this problem.

Injection systems often are based on a six-port valve, which is put through several steps for each injection, as illustrated in figure 46. In the first step, denoted here as *load*, the sample is either aspired by a vacuum (in automated systems) or expressed by a syringe plunger (in manual systems) into a sample loop, where it rests until the valve is switched to *inject*. This second step connects the pump and the mobile phase with the column. The contents of the sample loop then move into the solvent flow path and onto the analytical column. Because all parts of the system are constantly flushed during analysis, the remnants of a previous injection are removed before the next injection occurs.

The quality of the separation on the column depends on the quality of the injection—a short, sharp injection increases the likelihood of short, sharp peaks. The use of a minimum number of fittings between the injector and the column reduces the diffusion of the contents of the sample loop into the mobile-phase fronts in front of and behind the column. So-called low dead volume fittings with the minimum required internal capacity are available. These fittings have no “dead ends” or unnecessary spaces where solvent and sample can mix.

## Manual injectors

Simple manual injectors remain popular in some laboratories because they are inexpensive and because their operation requires little previous experience, which is important if the equipment is used infrequently. With a precision syringe, the operator can fill the sample loop at atmospheric pressure by injecting the contents into the injection port. A switch of the rotor attached to the valve realigns the valve ports to the *inject* position. Solvent from the pump then flushes the contents of the sample loop onto the column. Continual flushing during the run keeps the injection port and valve clear of remnants of previous samples.

| ✔ | ✘ |
|---|---|
| Inexpensive. | No automation and no provision for online derivatization. Syringe must be cleaned manually, offline. |

## Automated injectors

Automated injectors contain a mechanically driven version of the same six-port valve found in manual injectors. Pneumatic or electrical actuators control the valve as it switches between steps in the injection cycle. A metering device can handle injection volumes of 0.1–1500 µl. With sample loops of larger capacity, such a device can inject up to 5 ml. Vials designed to hold microliter volumes can be used to inject as little as 1 µl of a 5-µl sample. Even the way the needle enters the vial can be controlled with computer software: deep down to aspire from the denser of two layers, or a shallow dip into the supernatant phase. With this technique, even viscous samples can be analyzed if the right equipment is used. The time required to extract the syringe plunger is simply protracted, permitting meniscus motion of higher reproducibility.

| ✔ | ✘ |
| --- | --- |
| Highly reproducible. Can be fully automated. Flow maintained over all parts in contact with sample, preventing inaccuracies from intermingling between runs. | Equipment can be costly. |

## Autosampler with sample pretreatment capabilities

Autosamplers can provide online precolumn derivatization, dilute small volumes of sample, and add internal standards. You may need to protect unstable species by keeping the sample chilled with a cooling device connected to a flow of refrigerated water or, more conveniently, by Peltier elements. Alternatively, you might want to induce reactions using heat applied within the injection device of the autosampler. Commercially available autosamplers offer all these features.

## Derivatization

As discussed later in chapter 8, derivatization may be required if the analytes lack chromophores and if detection is not sensitive enough. In this process, a chromophore group is added using a derivatization reagent. Derivatization can occur either in front of or behind the analytical column and is used to improve sensitivity and/or selectivity. Precolumn derivatization is preferable because it requires no additional reagent pump and because reagents can be apportioned to each sample rather than pumped through continuously. Automated precolumn derivatization yields excellent precision. Moreover, it can handle volumes in the microliter range, which is especially important when sample volume is limited. The principles involved are illustrated in figure 47.

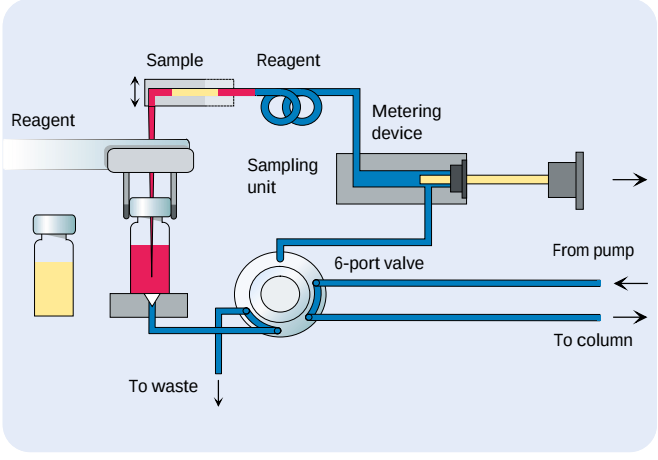


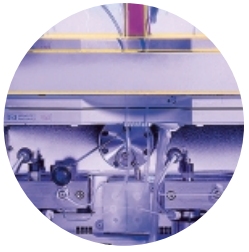

**Figure 47**
**Automated precolumn derivatization**

The robotic arm of the autosampler transports, in turn, a sample vial and several reaction vials under the injection needle. The needle is extended by a length of capillary at the point at which the derivatization reaction takes place.

The injector draws distinct plugs of sample and derivatization reagent into the capillary. The back-and-forth movement of the plunger mixes the plugs. With the right software, the autoinjector can be paused for a specified length of time to allow the reaction to proceed to completion. If the reaction requires several reagents, the autosampler must be programmable, that is, it must be able to draw sequentially from different reagent vials into one capillary.
In this complex sample manipulation, the needle must be cleaned between vials, for example by dipping into wash vials of distilled water.

**In brief…**

Automated sampling systems offer significant advantages over manual injectors, the most important of which is higher reproducibility of the injection volume. Sample throughput also can be increased dramatically. Modern autosamplers are designed for online sample preparation and derivatization. For food analysis, an automated injection system is the best choice.



*Chapter 7*

# Mobile phase pumps and degassers

**The pump is the most critical piece of equipment for successful HPLC. Performance depends strongly on the flow behavior of the solvent mixture used as mobile phase—varying solvent flow rates result in varying retention times and areas. Conclusions from a calibration run for peak identification or quantification depend on reproducible data. In this chapter we discuss multiple aspects of pump operation, including solvent pretreatment and its effect on performance.**

## Characteristics of a modern HPLC pump

A modern HPLC pump must have pulse-free flow, high precision of the flow rates set, a wide flow rate range, and low dead volume. In addition, it must exhibit control of a maximum operating pressure and of at least two solvent sources for mobile-phase gradients, as well as precision and accuracy in mixing composition for these gradients.

### Flow ranges

We discuss two gradient pump types: that constructed for flow rates between 0.2 and 10 ml/min (low-pressure gradient formation), and that designed for flow rates between 0.05 and 5 ml/min (high-pressure gradient formation).

### Gradient elution

In separating the multiple constituents of a typical food sample, HPLC column selectivity with a particular mobile phase is not sufficient to resolve every peak. Changing the eluant strength over the course of the elution by mixing increasing proportions of a second or third solvent in the flow path above the column improves peak resolution in two

ways. First, resolution is improved without extending the elution period, which prevents long retention times (peaks that have been retained on the column for a longer period of time tend to broaden and flatten through diffusion, lowering the S/N and therefore detection levels). Second, gradient elution sharpens peak widths and shortens run time, enabling more samples to be analyzed within a given time frame. The solvents that form the gradient in front of the column can be mixed either after the pump has applied high pressure or before, at low pressure.

### Gradient formation at high pressure

If mixing takes place after pressure has been applied, a high-pressure gradient system results (this is most often achieved by combining the output of two isocratic pumps, each dedicated to one solvent).

| ✔ | ✘ |
|---|---|
| Ability to form sharp gradient profiles and to change solvents rapidly (100% A to 100% B), without degassing, for standard applications. | Expensive. An additional mixer for lowest mixing noise at flow rates below 200 µl is needed for mobile-phase compositions. |

### Gradient formation at low pressure

At low pressure, mixing of the gradient solvents occurs early in the flow path before the pump applies pressure, as in the two examples below.

| ✔ | ✘ |
|---|---|
| Less expensive than gradient elution. Can mix more than two channels. Low mixing noise without a dedicated mixer. | Degassing is necessary for highest reproducibility. |

# Pump designs for gradient operation

### Low-pressure gradient Agilent 1100 Series pump

In food analysis, pump performance is critical. In the examples, we describe a low-pressure gradient system and a high-pressure gradient system, both of which perform according to food analytical requirements. The former has a single dual-piston mechanism for low-pressure gradient formation, whereas the latter has a double dual-piston mechanism for high-pressure gradient formation. After passing the online vacuum degasser, the mobile phase enters the first pump chamber through an electronically activated inlet valve (see figure 48). Active valves resolve the problem of contaminated or sticky ball valves by making the pump easy to prime. Output from the first piston chamber flows through a second valve and through a low-volume pulse dampener (with pressure transducer) into a second piston chamber. Output from the second chamber flows onto the sampling unit and column. The pistons in the pump chambers are motor driven and operate with a fixed-phase

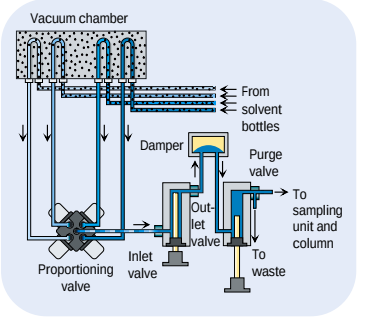



**Figure 48**
**Low pressure gradient pump**

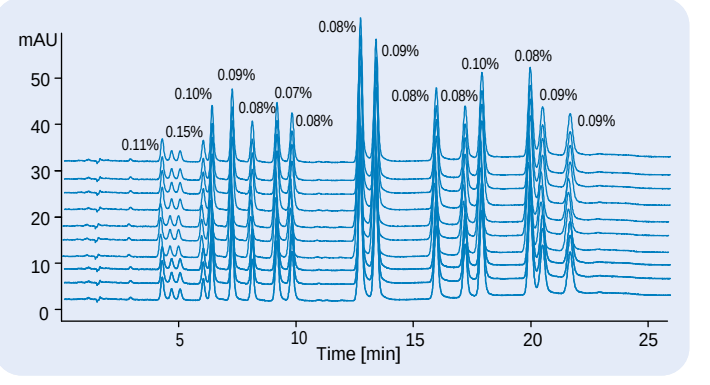



**Figure 49**
**Retention time precision (% RSD) of 10 injections of a polycyclic aromatic hydrocarbon (PNA) standard sample**

difference of 180°, so that as one delivers mobile phase, the other is refilling. The volume displaced in each stroke can be reduced to optimize flow and composition precision at low flow rates. With solvent compressibility, compensation, and a low-volume pulse dampener, pulse ripple is minimal, resulting in highly reproducible data for retention times and areas (see figure 49). A wide flow range of up to 10 ml/min and a delay volume of 800–1100 µl support narrow-bore, standard-bore, and semipreparative applications. Four solvents can be degassed simultaneously with high efficiency.

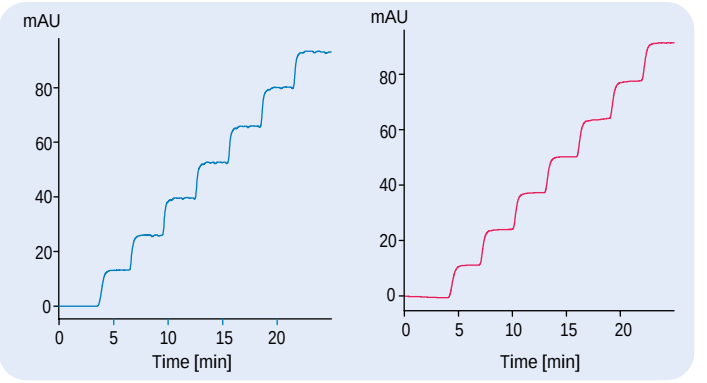



**Figure 50**
**Results of a step-gradient composition (0–7%) of a high-pressure pump (left) and of a low-pressure pump (right)**

In this design, gradients are formed by a high-speed proportioning valve that can mix up to four solvents on the low-pressure side. The valve is synchronized with piston movement and mixes the solvents during the intake stroke of the pump. The solvents enter at the bottom of each chamber and flow up between the piston and the chamber wall, creating turbulences. Compared with conventional multisolvent pumps with fixed stroke volumes, pumps with variable stroke volumes generate highly precise gradients, even at low flow rates (see figure 50).

| **Performance of low-pressure pump design** | |
|---|---|
| Flow precision | < 0.3 % (typically < 0.15 %) based on retention times of 0.5 and 2.5 ml/min |
| Flow range | 0.2–9.999 ml/min |
| Delay volume | ca. 800–1100 µl |
| Pressure pulse | < 2 % amplitude (typically < 1 %), 1 ml/min propanol, at all pressures |
| Composition precision | ± 0.2 % SD at 0.2 and 1 ml/min |

## High-pressure gradient Agilent 1100 Series pump

The Agilent 1100 Series high-pressure gradient pump is based on a double dual-piston mechanism in which two pumps are connected in series in one housing. This configuration takes up minimal bench space and enables very short internal and external capillary connections. Both pistons of both individual pumps are servocontrolled in order to meet chromatographic requirements in gradient formation (see figure 51).

Three factors ensure gradients with high precision at low flow rates: a delay volume as low as 180–480 µl internal volume (without mixer), maximum composition stability and retention time precision, and a flow range typically beginning at 50 µl/min.

The same tracer gradient used to determine composition precision and accuracy also was used to determine the ripple of the binary pump (see figures 50 and 52). The delay volume was measured by running a tracer gradient. Large delay volumes reduce the sharpness of the gradient and therefore the selectivity of an analysis. They also increase the run-time cycle, especially at low flow rates.

**Performance of high-pressure pump design**

| | |
|---|---|
| Flow precision | < 0.3 % |
| Flow range | 0.05–5 ml/min |
| Delay volume | 180–480 µl (600–900 µl with mixer |
| Pressure pulse | < 2 % amplitude (typically, 1 %), 1 ml/min isopropanol, at all pressure > 1MPa |
| Composition precision | < 0.2 % at 0.1 and 1.0 ml/min |

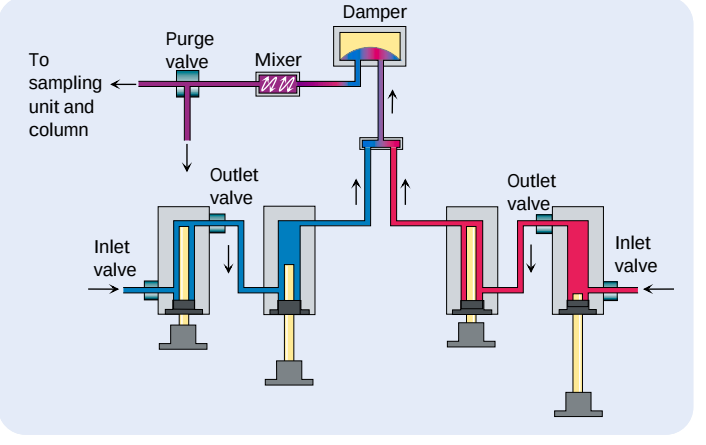



**Figure 51**
**Schematics of the high-pressure gradient Agilent 1100 Series pump**

When working at the lowest detection limits, it is important to use a mixer to reduce mixing noise, especially at 210–220 nm and with mobile phases containing solvents such as tetrahydrofuran (THF). Peptide mapping on 1-mm columns places stringent demands on the pump because small changes in solvent composition can result in sizeable changes in retention times. Under gradient conditions at a flow rate of 50 µl/min, the solvent delivery system must deliver precisely 1 µl/min per channel. A smooth baseline and nondistorted gradient profiles depend on good mixing and a low delay volume. Figure 53 shows six repetitive runs of a tryptic digest of myoglobin with a retention time precision of 0.07–0.5% RSD.

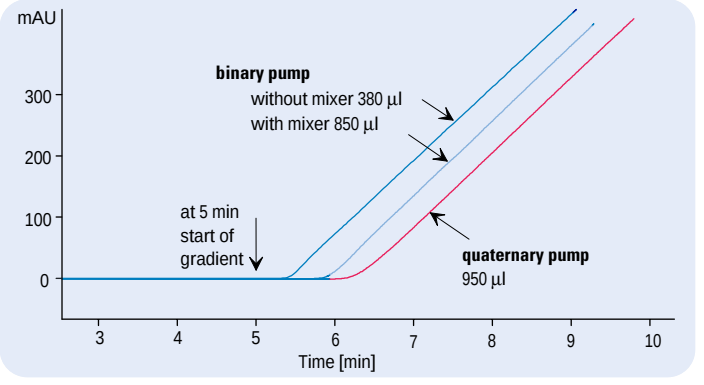



**Figure 52**
**Delay volume of high- and low-pressure gradient pumps**

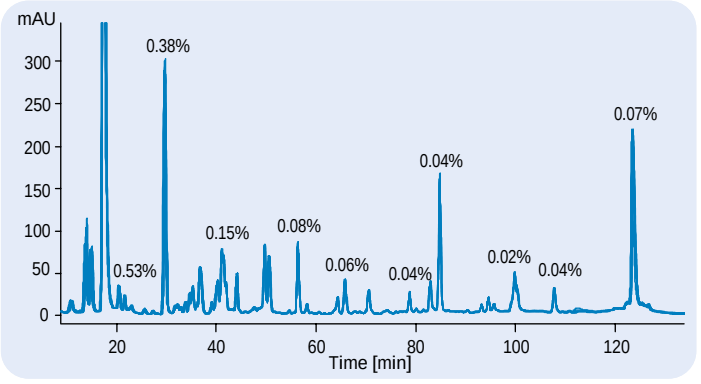



**Figure 53**
**Overlay of six repetitive runs of a tryptic digest of myoglobin in RSD of RT is as low as 0.07–0.5 %**

## Degassing

Degassing removes dissolved gases from the mobile phase before they are pumped over the column. This process prevents the formation of bubbles in the flow path and eliminates volumetric displacement and gradient mixing, which can hinder performance. Instable flow causes retention on the column and may increase noise and drift on some flow-sensitive detectors. Most solvents can partially dissolve gases such as oxygen and thereby harm detectors. Detrimental effects include additional noise and drift in UV detectors, quenching effects in fluorescence detectors, and high background noise from the reduction of dissolved oxygen in electrochemical detectors used in reduction mode (in oxidation mode, the effect is less dramatic).

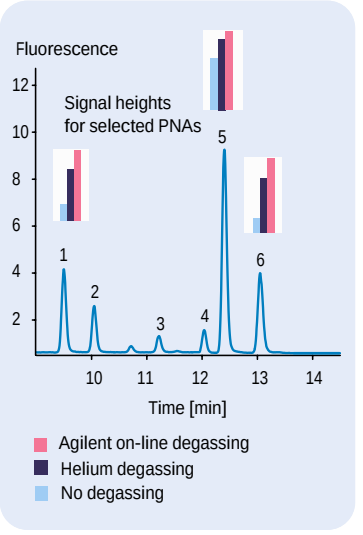



**Figure 54**
**The loss of response due to quenching can be recovered with either helium or vacuum degassing.**

The oxygen effect is most apparent in the analysis of polycyclic aromatic hydrocarbons (PNAs) with fluorescence detection, as shown in figure 50. The less oxygen present in the mobile phase, the less quenching occurs and the more sensitive the analysis.

In general, one of three degassing techniques is used: on- or offline vacuum degassing, offline ultrasonic degassing, or online helium degassing. Online degassing is preferable since no solvent preparation is required and the gas concentration is held at a constant, minimal level over a long period of time. Online helium and online vacuum degassing are the most popular methods.

## Helium degassing

In helium degassing, gas is constantly bubbled through the mobile-phase reservoir. This process saturates the solvent and forces other gases to pass into the headspace above.

| ✔ | ✘ |
|---|---|
| Requires only a simple regulator. Several channels can be purged simultaneously without additional dead volume. | Expensive. Evaporation of the more volatile components can change composition over time. Oxygen is better purged by vacuum degassing. |

### Vacuum degassing

In vacuum degassing, the solvent is passed through a membranous tube made of a special polymer that is permeable to gas but not to liquids under vacuum. The pressure differences between the inside and outside of the membrane cause continuous degassing of the solvent. New online degassers with low internal volume (< 1 ml) allow fast changeover of mobile phases.

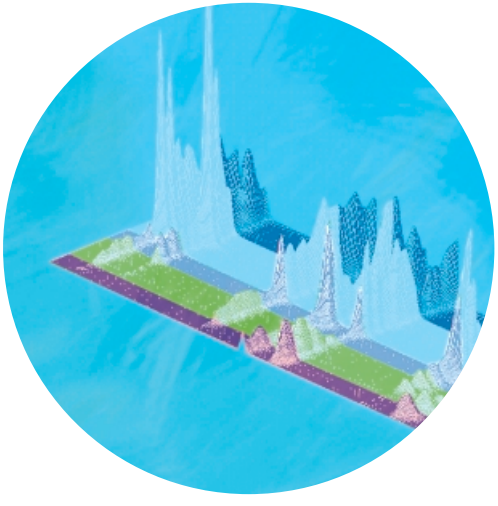
| ✔ | ✘ |
|---|---|
| Less expensive to use and maintain than helium degassing. The composition of premixed solvents is unaffected, and removal of oxygen is highly efficient. Several channels can be degassed simultaneously. | Increases dead volume and may result in ghost peaks, depending on the type of tubing and type of solvent used. |

## In brief…

The choice of pump depends on both elution mode (isocratic or gradient) and column diameter (narrow bore or standard bore). Although an isocratic system often is sufficient, gradient systems are more flexible. Moreover, their short analysis times make gradient systems ideal for complex samples, sharp peaks, resolution of multiple species, and automatic system cleansing with additional online solvent channel. Agilent 1100 Series pumps are best suited for flow ranges from 0.05 ml/min up to 10 ml/min and can therefore be used with columns that have an inner diameter of 1 mm to 8 mm. Although many officially recognized methods are based on standard columns and flow rates, the trend is toward narrow-bore columns. These consume less solvent, which also reduces waste disposal, thus lowering operating costs.

# *Chapter 8*

## Detectors



**Most detectors currently used in HPLC also can be applied in the analysis of food analytes. Each technique has its advantages and disadvantages. For example, diode array UV-absorbance detectors and mass spectrometers provide additional spectral confirmation, but this factor must be weighed against cost per analysis when deciding whether to use a detector routinely.**

The ability to use UV spectra to confirm the presence of certain food analytes and their metabolites and derivatives makes UV absorbance the most popular detection technique. However, for analytical problems requiring high sensitivity and selectivity, fluorescence detection is the method of choice. Although electrochemical detectors are also highly sensitive and selective, they are rarely used in food analysis. Conductivity detectors, on the other hand, are well-suited for the sensitive and selective analysis of cations and anions, and thermal energy detectors are used for high-sensitivity determination of nitrosamines down to 10 parts per trillion (ppt). Refractive index (RI) detectors are appropriate only if the above-mentioned detectors are not applicable or if the concentration of analytes is high, or both.

## Analytical parameters

The most important parameters for food analysis are:

- limit of detection (LOD) and limit of quantification (LOQ)
- linearity
- selectivity
- qualitative information

### Limit of detection and limit of quantification

The LOD and LOQ of an analytical system depend on the noise and drift of the detection equipment. Absolute detector LOD can be determined by injecting a sample directly into the detector. It is often expressed as minimum detectable level, which is sometimes defined as equal to the noise level. However, the LOD depends not only on the detector but may also depend on the oxygen content of the mobile phase, the injection system, peak broadening on the column, and temperature differences among system components. Taking these factors into account, the LOD is defined as 2 to 3 times the noise level. The LOQ is defined as 10 to 20 times the noise level. A UV detection system can be used to measure quantitative amounts down to 500 pg per injection. The LOD can be as low as 100 pg for food compounds such as antioxidants if detection wavelengths have been optimized to match the extinction coefficients of as many compounds as possible. Fluorescence and electrochemical detectors operate in the very low picogram range. The LOD of a mass spectrometer connected to HPLC equipment depends on the type of interface used. Instruments with electrospray interfaces can detect down to the picogram range. Refractive index detectors normally are appropriate above 500 ng.

### Selectivity

We define the selectivity of a detection system as the ability to select only those compounds of interest in a complex matrix using specific compound properties. A detector is selective if it does not respond to coeluting compounds that

could interfere with analyte quantification. A UV absorbance detector can be made selective by setting an appropriate wavelength with a narrow bandwidth for the compound of interest. However, the selectivity of detectors based on such a universal feature is low compared with the selectivity of detectors based on fluorescence and electrochemistry. Response characteristics are very selective, shown by a limited number of compounds. Mass spectrometers can be applied selectively or universally (in total scan mode), depending on the analysis to be performed. RI detectors are universal by definition.

**Linearity**

Detector response can be expressed both as dynamic range and as linear dynamic range. Dynamic range is the ratio of the maximum and the minimum concentration over which the measured property (absorbance, current, and so on) can be recorded. However, in practice, linear dynamic range—the range of solute concentration over which detector response is linear—is more commonly used. Plotting the response of injections of different analyte concentration against their concentrations should give a straight line over part of the concentration range. Response often is linear for only one tenth of the full dynamic range. UV detectors are linear over a range of a maximum of five orders of magnitude, whereas fluorescence and electrochemical detectors are linear over a range of two orders of magnitude. Mass spectrometers are usually linear over three orders of magnitude, and RI detectors are linear over a maximum of four orders of magnitude.

**Qualitative information**

A classical identification tool in chromatography is the mass spectrogram, which is recorded by a mass spectrometer. Its appeal in HPLC, however, is limited owing to the cost of interfacing the mass spectrometer equipment. If the spectra of the analytes differ markedly, UV absorbance spectra can be used for identification using diode array technology. Fluorescence and electrochemical detectors can be used only to identify compounds based on their retention times.

## UV detectors

Figure 55 shows the optical path of a conventional variable wavelength detector. Polychromatic light from a deuterium lamp is focused onto the entrance slit of a monochromator using spherical and planar mirrors. The monochromator selectively transmits a narrow band of light to the exit slit. The light beam from the exit slit passes through the flow cell and is partially absorbed by the solution in the flow cell. The absorbance of the sample is determined by measuring the intensity of the light reaching the photodiode without the sample (a blank reference) and comparing it with the intensity of light reaching the detector after passing through the sample.

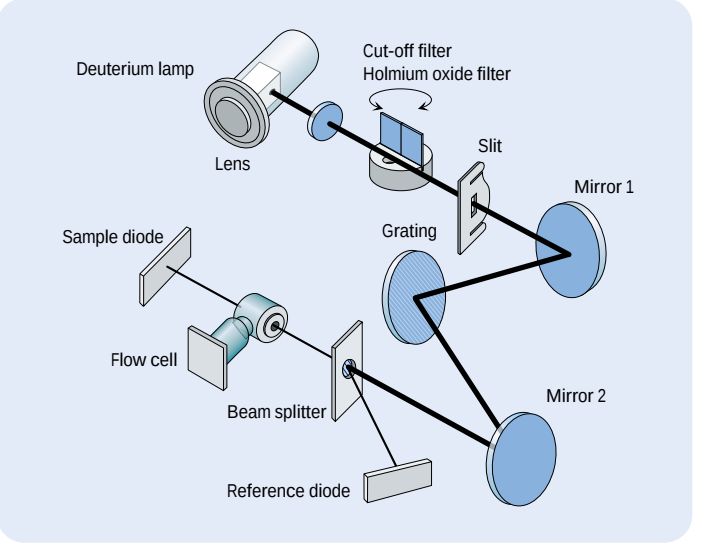



**Figure 55**
**Conventional variable wavelength detector**

Most variable wavelength detectors split off part of the light to a second photodiode on the reference side. The reference beam and the reference photodiode are used to compensate for light fluctuations from the lamp. For optimum sensitivity,

the UV detector can be programmed for each peak within a chromatographic run, which changes the wavelength automatically. The variable wavelength detector is designed to record absorbance at a single point in the spectrum at any given point in time. However, in practice, different wavelengths often must be measured simultaneously, for example when two compounds cannot be separated chromatographically but have different absorbance maxima. If the entire spectrum of a compound is to be measured, the solvent flow must be stopped in order for a variable wavelength detector to scan the entire range, since scanning takes longer than elution.

| ✔ | ✘ |
|---|---|
| Sensitive; can be tuned to the wavelength maxima of individual peaks. Some instruments are equipped with scanning mechanisms with stopped-flow operation. | Single-wavelength measurement is not always sufficient. Without spectra, peaks cannot be identified. |

## Diode array detectors

Figure 52 shows a schematic diagram of a photodiode array detector (DAD). An achromatic lens system focuses poly-

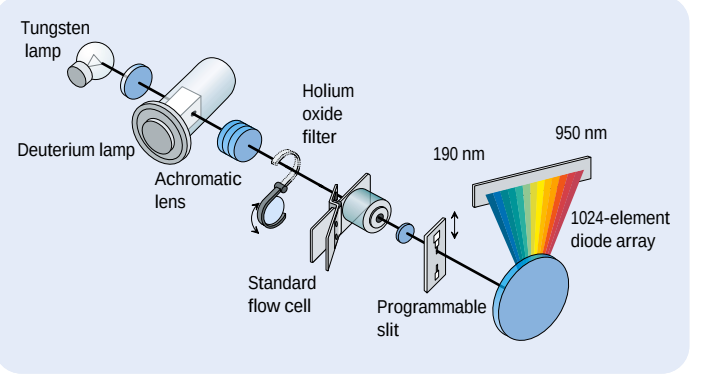



**Figure 56**
**Diode array detector optics**

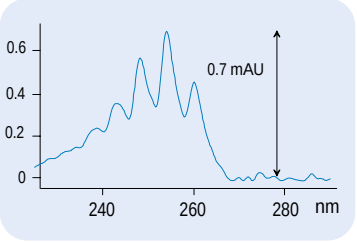



**Figure 57**
**High-resolution spectrum for benzene in the low absorbance range**

| | Conventional | DAD |
|---|---|---|
| **Signal acquisition** | 1 | 8 |
| **Spectra acquisition** | stop flow | on-line |

chromatic light from the deuterium and tungsten lamps into the flow cell. The light then disperses on the surface of a diffraction grating and falls on the photodiode array. The range varies from instrument to instrument. The detector shown here is used to measure wavelengths from 190 to 950 nm using the twin-lamp design.

In our example, the array consists of 1024 diodes, each of which measures a different narrow-band spectrum. Measuring the variation in light intensity over the entire wavelength range yields an absorption spectrum. The bandwidth of light detected by a diode depends on the width of the entrance slit. In our example, this width can be programmed to selected values from 1 to 16 nm. If very high sensitivity is required, the slit is opened to 16 nm for maximum light throughput. If maximum spectral resolution is needed, the slit is narrowed to 1 nm. At this setting, the fine structure of benzene can be detected, even at 0.7 mAU full-scale (mAUFS; see figure 57). Because the relative positions of the sample and the diffraction grating are reversed compared with a conventional instrument, this configuration is often referred to as reversed optics. The most significant differences between a conventional UV absorbance detector and a DAD are listed at left.

### Three dimensions of data

DADs connected to appropriate data evaluation units help optimize wavelengths for different compounds over the course of the run. Maxima can be seen easily using three-dimensional plots of data, or as absorbance intensity plotted over time at different wavelengths, that is, as an isoabsorbance plot (see figure 58). Figure 55 illustrates the optimization result for antibiotics. The ability to acquire and store spectra permits the creation of electronic spectral libraries, which can be used to identify sample compounds during method development.

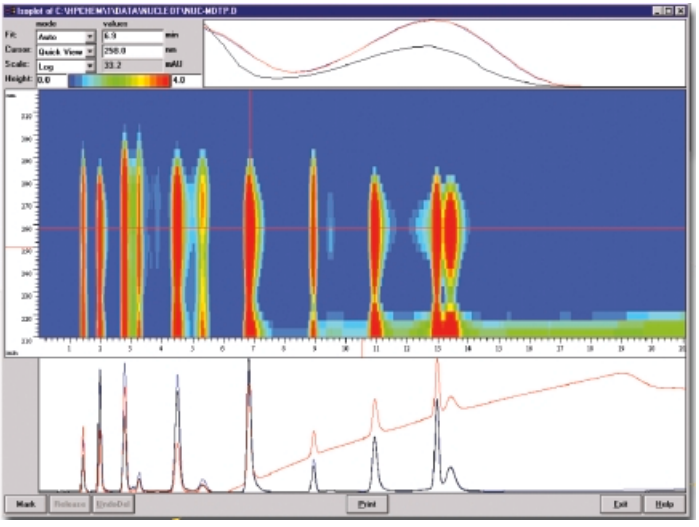


**Figure 58**
**Isoabsorbance plot**

Multisignal detection yields optimum sensitivity over a wide spectral range. However, the spectral axis in figure 58 shows that no single wavelength can detect all antibiotics at highest sensitivity.

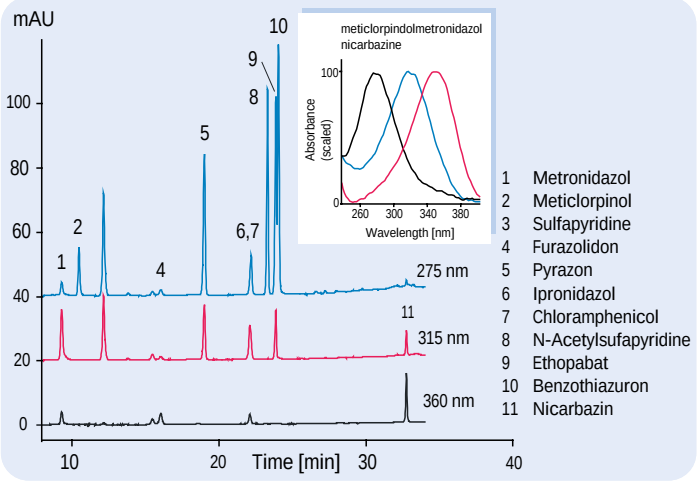



**Figure 59**
**Multisignal detection of antibiotic drugs**

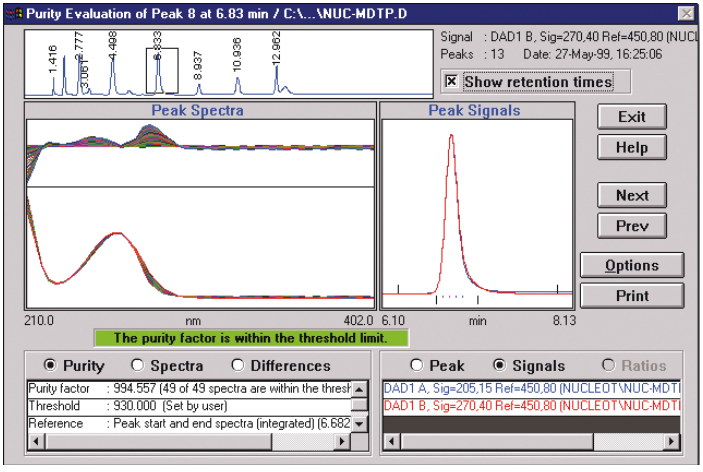
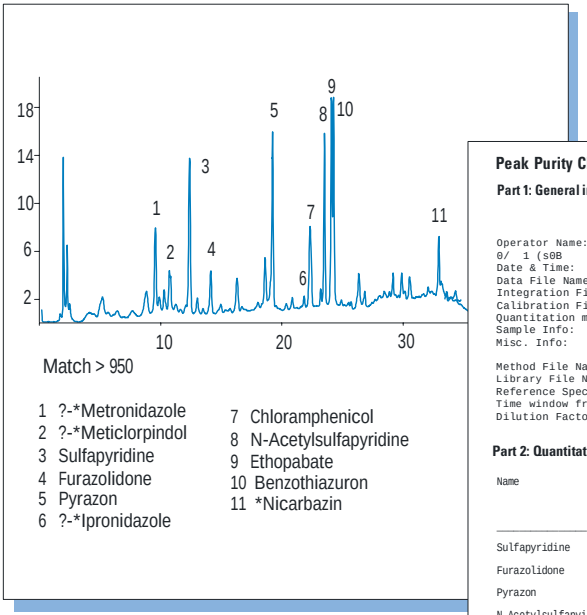



**Figure 60**
**Peak purity analysis**

In light of the complexity of most food samples, the ability to check peak purity can reduce quantification errors. In the most popular form of peak purity analysis, several spectra acquired during peak elution are compared. Normalized and overlaid, these spectra can be evaluated with the naked eye, or the computer can produce a comparison. Figure 60 shows a peak purity analysis of antibiotics. If a spectral library has been established during method development, it can be used to confirm peak identity. Analyte spectra can be compared with those stored in the library, either interactively or automatically, after each run.

Figure 61 shows both the quantitative and qualitative results of the analysis. Part one of this primer contains several applications of UV absorbance DAD detection.




**Peak Purity Check and Identification**

**Part 1: General information**

* * * * * REPORT * * * * *

```
Operator Name:          BERWANGER                    (s1B        Vial/Inj.No.:
0/  1 (s0B
Date & Time:            10 Sep 86   9:17 am
Data File Name:         LH:LETAA00A
Integration File Name:  DATA:DEFAULT.I
Calibration File Name:  DATA:ANTI.Q
Quantitation method:    ESTD                         calibrated by     Area response
Sample Info:            DOTIERUNGSVERSUCHE
Misc. Info:

Method File Name:       ANTIBI.M                     Wavelength from: 230 to: 400 nm
Library File Name:      DATA:ANTIBI.L                Library Threshhold:         950
Reference Spectrum:     Apex                         Peak Purity Threshold:      950
Time window from:       6.0 % to: 2.0 %              Smooth Factor:                7
Dilution Factor: 1.0    Sample Amount: 0.0           Resp.Fact.uncal.peaks:     None
```

**Part 2: Quantitation, peak purity check and peak identification**

| Name | | Amount [ng/l] | | Peak-Ret. [min] | Cal.-Ret. [min] | Lib.-Ret [min] | Purity | Library | Res. Matchfactor |
|---|---|---|---|---|---|---|---|---|---|
| Sulfapyridine | | 10.31 | A | 12.183 | 12.143 | 12.159 | 999 | 1000 | 0.9 |
| Furazolidone | | 4.54 | A | 16.096 | 16.024 | 16.028 | 992 | 984 | 1.3 |
| Pyrazon | | 13.72 | A | 19.024 | 18.987 | 19.000 | 1000 | 1000 | 1.7 |
| N-Acetylsulfapyidine | | 14.66 | A | 23.307 | 23.282 | 23.282 | 976 | 1000 | 1.1 |
| Ethopabat | *up | 13.40 | A | 23.874 | 23.840 | 23.848 | 911 | 996 | 2.3 |
| Benzthiazuron | | 12.80 | A | 24.047 | 24.024 | 24.029 | 998 | 1000 | 0.7 |
| Nicarbazin | *up | 3.00 | A | 32.733 | 32.722 | 32.709 | 336 | 984 | 1.2 |
| | | ======== | | | | | | | |
| | | 72.41 | | | | | | | |

**Figure 61**
**Quantitative and qualitative results for the analysis of antibiotic drugs**

| ✔ | ✘ |
|---|---|
| Enables maximum peak purity and identity, measurement of multiple wavelengths, acquisition of absorbance spectra, and spectral library searches. | DADs are best suited for universal rather than sensitive analysis (for which electrochemical or fluorescence detection is more appropriate). |

## Fluorescence detectors

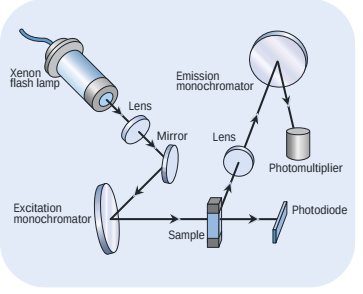



**Figure 62**
**Schematics of a fluorescence detector**

Fluorescence is a specific type of luminescence that is created when certain molecules emit energy previously absorbed during a period of illumination. Luminescence detectors have higher selectivity than, for example, UV detectors because not all molecules that absorb light also emit it. Fluorescence detectors are more sensitive than absorbance detectors owing to lower background noise. Most fluorescence detectors are configured such that fluorescent light is recorded at an angle (often at a right angle) to the incident light beam. This geometry reduces the likelihood that stray incident light will interfere as a background signal and ensures maximum S/N for sensitive detection levels.

The new optical design of the Agilent 1100 Series fluorescence detector is illustrated in figure 62. A Xenon flash lamp is used to offer the highest light intensities for excitation in the UV range. The flash lamp ignites only for microseconds to provide light energy. Each flash causes fluorescence in the flow cell and generates an individual data point for the chromatogram. Since the lamp is not powered on during most of the detector operating time, it offers a lifetime of several thousand hours. No warmup time is needed to get a stable baseline. A holographic grating is used as a monochromator to disperse the polychromatic light of the Xenon lamp. The desired wavelength is then focused on the flow cell for optimum excitation. To minimize stray light from the excitation side of the detector, the optics are configured such that the emitted light is recorded at a 90 degree angle to the incident light beam. Another holographic grating is used as the emission monochromator. Both monochromators have optimized light throughput in the visible range.

A photomultiplier tube is the optimum choice to measure the low light intensity of the emitted fluorescence light. Since flash lamps have inherent fluctuations with respect to flash-to-flash intensity, a reference system based on a

photodiode measures the intensity of the excitation and triggers a compensation of the detector signal.

**Cut-off filter**

Since the vast majority of emission maxima are above 280 nm, a cut-off filter (not shown) prevents stray light below this wavelength to enter the light path to the emission monochromator. The fixed cut-off filter and bandwidth (20 nm) avoid the hardware checks and documentation work that is involved with an instrument that has exchangeable filters and slits.

**Signal/spectral mode**

The excitation and emission monochromators can switch between signal and spectral mode. In signal mode they are moved to specific positions that encode for the desired wavelengths, as with a traditional detector. This mode offers the lowest limits of detection since all data points are generated at a single excitation and emission wave length.

**Online spectral measurements and multisignal acquisition**

A scan of both the excitation and the emission spectra can be helpful in method development. However, only detectors with motor-driven gratings on both sides can perform such a scan. Some of these detectors also can transfer this data to a data evaluation computer and store spectra in data files. Once the optimum excitation and emission wavelength has been determined using scanned spectra, detectors with grating optics can be programmed to switch between these wavelengths during the run.

The spectral mode is used to obtain multi-signal or spectral information. The ignition of the flash lamp is synchronized with the rotation of the gratings, either the excitation or emission monochromator. The motor technology for the gratings is a long-life design as commonly used in high-speed PC disk drive hardware. Whenever the grating has reached the correct position during a revolution, the Xenon lamp is ignited to send a flash. The flash duration is below two microseconds while the revolution of the grating takes less than 14 milliseconds. Because of the rotating monochromators, the loss in sensitivity in the spectral

mode is much lower compared to conventional dual-wavelength detection with UV detectors.

**Multisignal**

PNA analysis, for example, can be performed with simultaneous multi wavelength detection instead of wavelength-switching. With four different wavelengths for emission, all 15 PNAs can be monitored (figure 63).

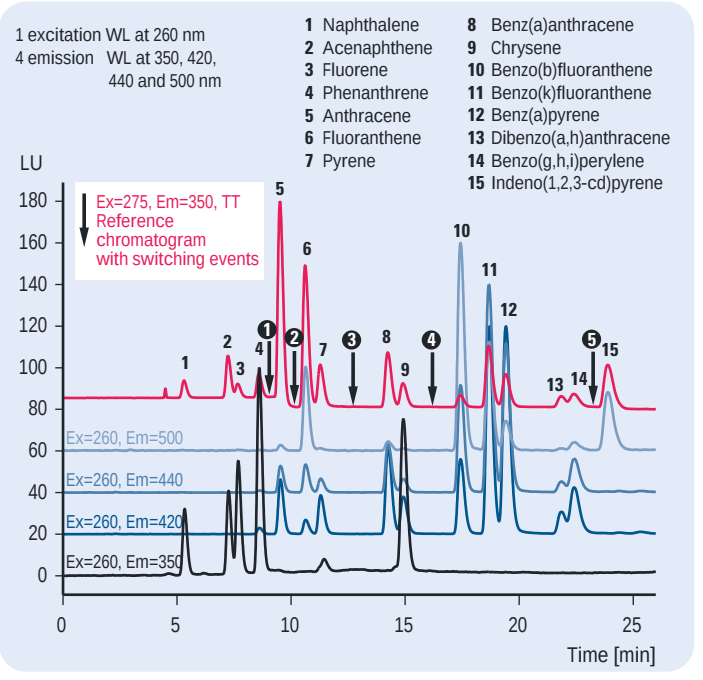



**Figure 63**
**Simultaneous multi wavelength detection for PNA-analysis**
**The upper trace was received with traditional wavelength switching.**
**❶ Ex/Em = 260/420 nm**
**❷ Ex/Em = 270/440 nm**
**❸ Ex/Em = 260/420 nm**
**❹ Ex/Em = 290/430 nm**
**❺ Ex/Em = 250/550 nm**

| ✔ | ✘ |
|---|---|
| Highly specific. Flash lamps eliminate drawback of baseline drift from heat transfer. Fluorescent tagging improves detection limits. | Fluorescence spectra are not commonly used to confirm peak identity. |

## Electrochemical detectors

Electrochemical detection techniques are based on the electrical charge transfer that occurs when electrons are given up by a molecule during oxidation or absorbed by a molecule during reduction. This oxidation or reduction takes place on the surface of a so-called working electrode. Whether a compound is reduced or oxidized and the speed of the reaction depend on the potential difference between the working electrode and the solution containing the compounds. From the activation energies and redox potentials expressed by the Nernst equation, reaction speed can be determined. The resulting current is proportional to the number of reactions occurring at the electrode, which in turn is an indicator of the concentration of the compound of interest at the surface.

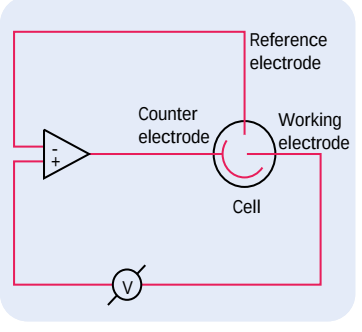



**Figure 64**
**Three-electrode electrochemical detector**

In the detection process, three electrodes are used: the working electrode, in which the reaction takes place; the counter electrode, which applies the potential difference between mobile phase and the working electrode; and the reference electrode, which compensates for any change in eluant conductivity (see figure 64). The reference electrode readings feed back to the counter electrode in order to keep the potential difference constant during peak elution as current flows through the working electrode.

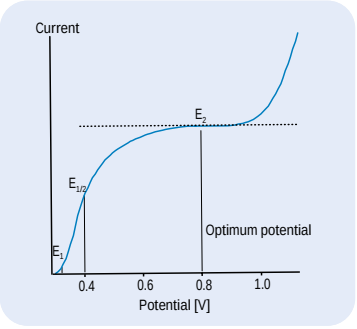



**Figure 65**
**Current-voltage relationship**

Detector response results from amplification of the electron flow and its subsequent conversion to a signal. Extremely low currents representing analyte quantities in the picogram range and below can be measured with today's advanced electronics. Although electrochemical detection can detect only those substances that can be electrolyzed, this limitation is actually an advantage when applied to complicated food matrices because it improves selectivity. To determine the optimum working electrode potential, the relationship between detector response (current) and potential applied (voltage) must be plotted for each compound as a current-voltage (CV) curve, as shown in figure 65. At a potential less than E1, oxidation cannot occur because the supply of energy is insufficient. Increasing the potential to E1/2 will electrolyze 50 % of all molecules at the surface of the electrode. Maximum response requires a potential just above E2. This potential is known as the limiting current because any further increase in voltage will limit detection by raising noise.

### Electrode materials

Several materials are used in working electrodes, the most common of which is glassy carbon. These materials also include gold (for sugars and alcohols), platinum (for chlorite, sulfite, hydrazine, and hydrogen peroxide), silver (for halogens), copper (for amino acids), mercury (in reductive mode for thiosulfate), and combined mercury-gold (in reductive mode for nitrogenous organic compounds).

### Flow cell aspects

Numerous cell designs have been described in the literature. The majority can be classified as one of three principal types: thin-layer design, wall-jet design, and porous flow-through design (see figure 66). The porous flow-through cell design differs significantly from the other two in that coulometric detection ensures 100 % reaction yield on the surface of the electrode. The other designs allow an efficiency of only 1–10 % by amperometric detection. However, amperometric detection is usually the more sensitive technique and is preferred over coulometric

detection. electrochemical detectors can employ 1-µl flow cells and are well-suited to narrow-bore HPLC.

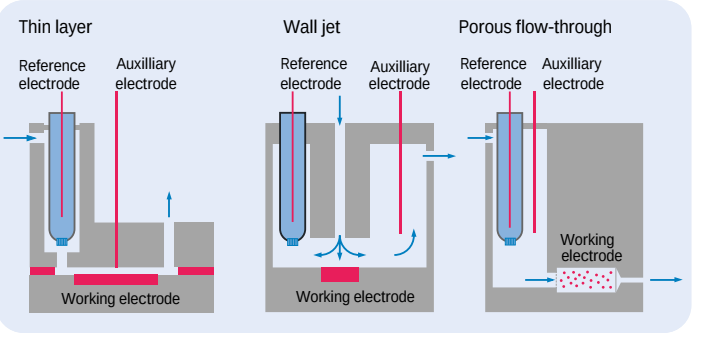



**Figure 66**
**Thin-layer design, wall-jet design, and porous flow-through design**

## Automation features

Until recently, the electrochemical technique was considered difficult to apply and not stable enough for routine analysis. However, recent improvements have made the use of these detectors routine, for example in the analysis of catecholamines in clinical research and routine testing laboratories. When applied between runs or even during peak elution (for example in sugar analysis using gold electrodes), self-cleaning routines based on pulsed amperometry improve stability (see figure 67).

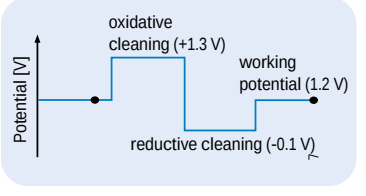



**Figure 67**
**Cleaning of working electrode**

Although an optimum potential for a mixture of compounds can be determined by evaluating the voltamograms for each compound, these optimizing steps can be automated using certain electrochemical detectors in so-called auto-increment mode. The HPLC equipment runs a series of injections over a range of increasing potentials (defined by start and end potentials and increment parameter), as shown in figure 68. A drift sensor helps ensure that a specified threshold is maintained before the next analysis begins (see figure 69).

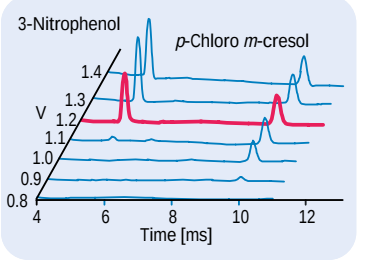



**Figure 68**
**Autoincrement mode**

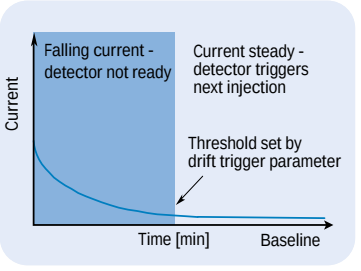



**Figure 69**
**Drift trigger**

Should the electrode surface of the flow cell become severely contaminated, as is likely for food matrixes, the cell must be disassembled and the electrode removed and cleaned in a strong acid or other suitable cleaning agent. Modern detectors are designed for ease of access and disassembly. Part one of this primer contains several applications of electrochemical detection.

## Mass spectrometers

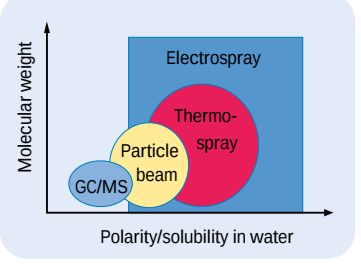



**Figure 70**
**Suitability of MS interfaces**

The identification of complex samples presents a problem for LC analysis. Coeluting compounds generally can be identified using UV absorbance detection with diode array technology, but this method may not be specific enough where spectra differences are low. Detection techniques such as fluorescence may offer higher specificity than UV detection, but if many different compounds are to be analyzed, these techniques also may not yield desired results. With mass spectroscopy (MS), several different analyte classes in a wide variety of sample types can be identified with greater certainty. Although GC/MS is a well-established technique for food analysis, LC/MS is only now emerging as a useful tool in this area. A GC-based analysis is appropriate only for those food compounds that are volatile and thermally stable (see figure 70). However, many compounds are nonvolatile, extremely polar, or thermally labile. Such compounds often can be separated successfully with LC, and the development of improved interfaces has made LC/MS more popular.

| HPLC | MS |
|---|---|
| High pressure liquid phase separation | High vacuum required |
| Produces large quantities of volatilized solvent (100–1000 ml/min gas)* | Typical MS vacuum systems designed for low ml/min gas load |
| No mass range limitation | Depends on masss/charge and mass range of analyzer |
| Can use inorganic buffers | Prefers volatile buffers |

* About 1000-fold increase going from liquid to gas phase with typical LC solvent

Mass spectrometers nevertheless are more easily interfaced with GC equipment than with LC equipment. The table at left lists the different operational conditions of LC and MS. Early efforts to interface LC with MS used direct liquid injection and moving-belt interfaces, but these methods proved ineffective and unreliable. In the 1980s, thermospray and particle beam interfaces improved both the range of applicability and the reliability of LC/MS. However, low sensitivity, the narrow mass and polarity range of analytes, and frequent maintenance requirements limited the effectiveness of these interfaces. More recently, two atmospheric pressure ionization (API) interfaces—electrospray and atmospheric pressure chemical ionization (APCI)—have replaced almost completely thermospray and particle beam techniques. These interfaces have a broad range of analyte molecular weights and polarities, high sensitivity, improved usability, and reduced maintenance needs. Selection of the appropriate LC/MS interface for an application depends on factors such as the polarity, molecular weight, and thermal lability of the analyte.

## API interfaces

In electrospray, effluent is directed through a nebulizing needle into a high-voltage field where charged droplets are formed (see figure 71). The charged droplets are then dried

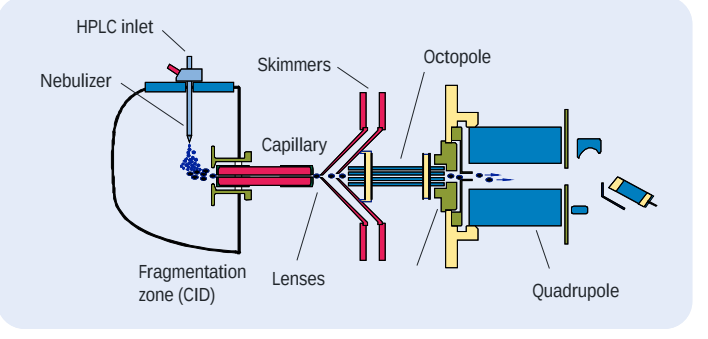



**Figure 71**
**API-electrospray LC/MS interface**

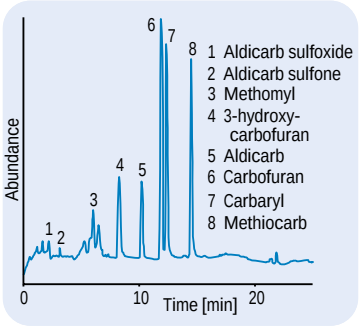



**Figure 72**
**Carbamate analysis**

and, as they shrink, analyte ions are desorbed. The ions are transported to the mass analyzer through a series of vacuum stages and ion-focusing elements.

Electrospray ionization can produce multiply charged ions of macromolecular analytes such as proteins and peptides. Because mass analyzers separate ions based on mass-to-charge ratio (m/z), lower-cost mass spectrometers with mass ranges of several thousand m/z can be used to analyze compounds in excess of 150,000 daltons. The primary use of electrospray has been the analysis of compounds of higher molecular weight. However, this technique also has been applied successfully to small polar molecules. Fig. 72 shows a separation of carbamate pesticides using electrospray.

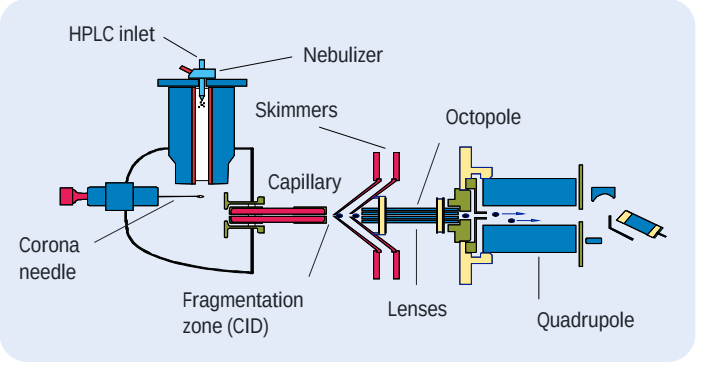



**Figure 73**
**APCI LC/MS interface**

APCI also can be used to analyze moderate polarity analytes. As in electrospray, APCI ionization occurs at atmospheric pressure via a chemical ionization process (see figure73).

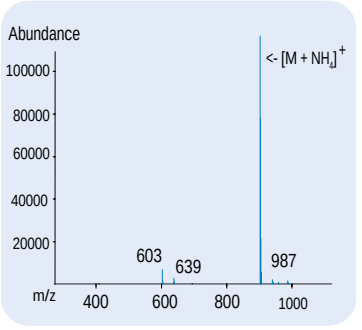



**Figure 74**
**Mass spectrum of the fatty acid triolein (C18:1, [cis]-9)**
**molecular weight = 884.781**
**molecular formula = $C_{57}H_{104}O_6$**

APCI requires some compound volatility and is less suitable for highly thermally labile compounds. Figure 74 shows a typical triglyceride mass spectrum. Both the degree of unsaturation and the length of the fatty acid side chains can be determined from the $[M + NH_4]$ + ion, which corresponds to mass M + 18. In-source CID experiments also can be helpful in determining the fatty acid composition of chromatographic peaks. Full-scan methods allow easy identification at the low nanogram level. If more precise quantitation is required, selected ion mode (SIM) can be used to obtain detection limits at the low picogram level.

| ✔ | ✘ |
|---|---|
| Polar and semipolar compounds up to 150,000 daltons can be analyzed. Highly sensitive. Strong molecular ions. Fragments, depending on in-source CID parameters. | Data analysis for complex heterogeneous mixtures of multiply charged analytes is not straightforward. Matrix can interfere with the ionization process. |

## Refractive index detectors

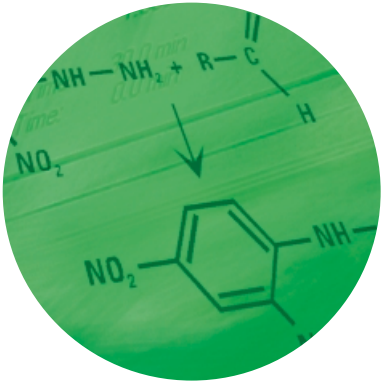
Refractive index (RI) detection is based on the difference in RI between the solution in the sample cell and the pure mobile-phase solution in the reference cell. Because the composition of the eluents must remain fixed throughout the analysis, this detector is not suitable for gradient analysis. Four main types of RI detectors are available: deflection according to Snell's law, reflection according to Fresnel's law, interference, and Christiansen effect. The first, which uses the dual-cell design, is by far the most popular. However, the nearly designed Agilent 1100 Series refractive index detector allows detection limits to the low ng range. Because RI detectors lack sensitivity and exhibit a tendency to drift owing to temperature changes, they are used primarily in the analysis of carbohydrates and nonaromatic acids.

| ✔ | ✘ |
|---|---|
| Universal detector. | Low sensitivity, no gradient operation. |

## In brief…

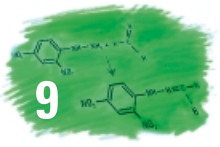
The following table reviews the detection techniques discussed in this chapter—your decision ideally should reflect a balance between desired results and financial resources.

| Detector | Sensitivity | Selectivity | Advantages | Applications |
|---|---|---|---|---|
| UV variable wavelength | + | - | Low cost, universal acids | Organic acids, fatty after derivatization, inorganic anions |
| UV-DAD | + | + | Peak purity confirmation | Antioxidants, preservatives, flavors, colorants, antiparasitic drugs, mycotoxins, pesticides, vitamins, amines after derivatization |
| Fluorescence | ++ | + | High sensitivity | Artificial sweeteners, mycotoxins, vitamins, carbamates, glyphosate |
| Electro-chemical | ++ | + | High sensitivity | Vitamins, inorganic anions |
| Mass spectro-meter scan | - | ++ | Identity, structure | Carbamates, lipids |
| Mass spectro-meter SIM | ++ | ++ | High selectivity | Pesticides, proteins |
| RI | - | - | Universal | Carbohydrates, nonaromatic acids |



*Chapter 9*

# Derivatization chemistries



# 9

**When analyte concentrations are particularly low, sample handling equipment for chemical derivatization can enhance the sensitivity and selectivity of results. As discussed in chapter 6, such equipment is available both pre- and postcolumn. In this chapter, we detail the chemistries that can be applied to food compounds and list the detection techniques for which they are best suited.**

## Addition of UV-visible chromophores

Labeling compounds with reagents that enable UV absorption is one of the most popular derivatization techniques. The reagent should be selected such that the absorption maximum of the reaction product exhibits not only improved sensitivity but also good selectivity. This combination reduces matrix effects resulting from the reagent, from by-products, or from the original matrix. The following table lists common compounds and reactions. In part one of this primer we give examples of compound derivatization, including that of fatty acids and amino acids.

| Target compound | | Reagent | λ |
|---|---|---|---|
| Alcohols | -OH | phenylisocyanate | 250 nm |
| Oxidizable sulfur compounds | $SO^3_2$- | 2,2'-dithiobis (5-nitro-pyridine) | 320 nm |
| Fatty acids | -COOH | p-bromophenacyl bromide<br>2-naphthacyl bromide | 258 nm<br>250 nm |
| Aldehydes and ketones | -CO-COOH, =C=O, and -CHO | 2,4-dinitrophenyl hydrazine | 365 nm |
| Primary amines | $-NH^2$ | o-phthalaldehyde (OPA) | 340 nm |
| Primary and secondary amines | NHR | 9-fluorenylmethyl chloroformate (FMOC) | 256 nm |

## Addition of a fluorescent tag

Fluorescence is a highly sensitive and selective detection technique. Adding fluorescent properties to the molecule of interest is of particular benefit in food analysis, in which components must be detected at very low concentrations. The following table lists common fluorescent tags. In part one of this primer we give examples for carbamates[41] and glyphosate.[42]

| Target compound | | Tagging reagent | λ |
|---|---|---|---|
| Alcohols | -OH | phenylisocyanate | λex 230 nm, λem 315 nm |
| Primary amines | $-NH^2$ | o-phthalaldehyde (OPA) | λex 230 nm, I em 455 nm |
| Primary and secondary amines | NHR | 9-fluorenylmethyl, chloroformate (FMOC) | λex 230 nm, I em 315 nm |

## Precolumn or postcolumn?

Precolumn techniques can be run either offline or online, but postcolumn techniques should be run online for maximum accuracy. In postcolumn derivatization, reagents can be added only through supplementary equipment (see figure 75) such as pumps. Mixing and heating devices also may be required. Increasing the dead volume behind the column in this way will result in peak broadening. Although this broadening may have no effect on standard-bore columns with flow rates above 1 ml/min, postcolumn derivatization is not suitable for narrow-bore HPLC.

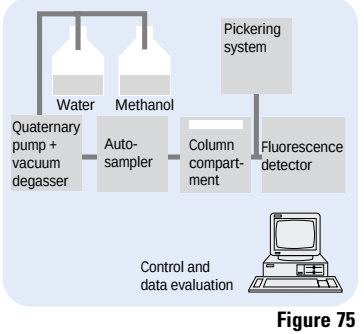
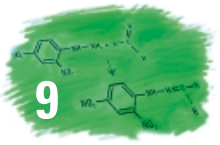



**Figure 75**
**Pickering postcolumn derivatization equipment for the analysis of carbamates**



9

## Automatic derivatization

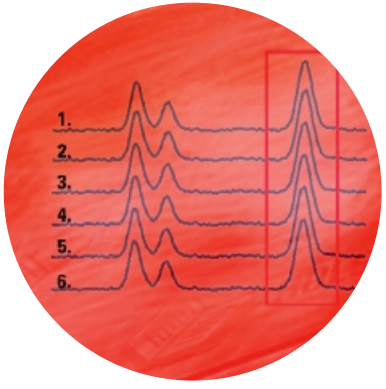
| | | | |
|---|---|---|---|
| 1 | Draw | 1.0 µl | from vial 12 |
| 2 | Draw | 0 µl | from vial 0 |
| 3 | Draw | 1.0 µl | from vial 8 |
| 4 | Draw | 0 µl | from vial 0 |
| 5 | Draw | 1.0 µl | from sample |
| 6 | Draw | 0 µl | from vial 0 |
| 7 | Mix | 8 cycles | |
| 8 | Draw | 1.0 µl | from vial 12 |
| 9 | Inject | | |

Both pre- and postcolumn derivatization techniques can be automated with modern HPLC equipment. The single-step mechanical functions of an autoinjector or autosampler can be programmed prior to analysis and stored in an injector program (see left). These functions include aspiration of the sample and of the derivatization agent, and mixing. Precolumn derivatization is fully compatible with narrow-bore HPLC and can result in fivefold improvements in S/N, with much lower solvent consumption than that from standard-bore methods. The analysis of fatty acids in part one of this primer illustrates this principle.

| ✔ | ✘ |
|---|---|
| Derivatization improves detectability of trace species. It can be automated and integrated online within the analysis. Many chemistries have been developed for routine use both pre- and postcolumn. | Additional investment in equipment. |

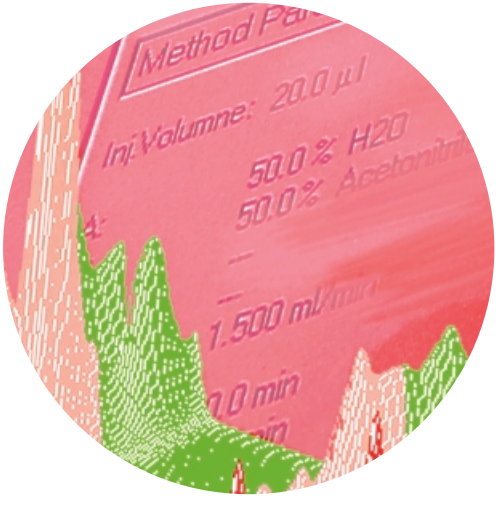
## In brief…

Derivatization offers enhanced analytical response, which is of benefit in food analysis. Chemical modifications can be automated either before or after separation of the compounds under study. In precolumn derivatization, autoinjectors with sample pretreatment capabilities (see chapter 6) are used, whereas in postcolumn derivatization, additional reagent pumps are plumbed to the chromatograph upstream of the detector. The latter approach adds dead volume and therefore is not suitable for the narrow-bore column technique described in chapter 4.



# *Chapter 10*

## Data collection and evaluation techniques



**Regardless which detection system you choose for your laboratory, the analytical data generated by the instrument must be evaluated. Various computing equipment is available for this task. The costs depend on the reporting requirements and on the degree of automation required.**

Depending on individual requirements, increasingly complex techniques are available to evaluate chromatographic data: at the simplest level are strip chart recorders, followed by integrators, personal computer–based software packages and, finally, the more advanced networked data systems, commonly referred to as NDS. Although official methods published by the U.S. Environmental Protection Agency (EPA) and by Germany's Deutsche Industrienorm (DIN) provide detailed information about calculation procedures and results, they give no recommendations for equipment.

## Strip chart recorders

Strip chart recorders traditionally have been used in connection with instruments that record values over a period of time. The recorder traces the measurement response on scaled paper to yield a rudimentary result. In the age of electronic data transfer, such physical records have been largely surpassed by data handling equipment preprogrammed to make decisions, for example to reject peaks that lie outside a certain time window.

| ✔ | ✘ |
|---|---|
| Inexpensive. | No record of retention times, no quantitative results on-line, no automatic baseline reset between runs, no electronic storage. |

## Integrators

Integrators offer several advantages over strip chart recorders and consequently are becoming the minimum standard for data evaluation. Integrators provide a full-scale chromatographic plot and multiple report formats. Area percent, normalization, and external and internal standard calculations are basic features of almost all modern integrators. Annotated reports list amounts, retention times, calculation type (peak areas or heights), and integration parameters as well as the date and time of measurement. Advanced features may provide for automated drawing of the baselines during postrun replotting and for the plotting of calibration curves showing detector response. For unattended analyses in which several runs are performed in series, integrators normally are equipped with a remote control connected to the autosampler in the system. Most models can also store raw data for replotting or reintegration at a later date. Some instruments have computer programming capabilities and can perform more advanced customized statistical calculations using the BASIC programming language, for example. Multichannel integrators are available for some analytical methods requiring two or more detection signals.

| ✔ | ✘ |
| --- | --- |
| Inexpensive. Facilitates reporting of retention times, quantitative results, and automatic baseline resets. | No instrument control or report customization. |

## Personal computers

In recent years personal computers (PCs) have become increasingly popular as data analysis tools in analytical laboratories. PCs offer more flexibility and better data storage capabilities than traditional storage methods. Moreover, on-line functions such as word processing, spreadsheet analyses, and database operations can be performed simultaneously (see figure 76). Through computer networks, laboratory instruments can be interconnected to enable the central archival of data and the sharing of printer resources. Client/server-based software extends these capabilities by distributing the processing across multiple processing units and by minimizing the time spent validating software.
With PCs, all aspects of the HPLC system can be accessed using a single keyboard and mouse. Parameters for all modules, including pump, detector, and autosampler, can be entered in the software program, saved to disk, and printed for documentation. Some HPLC software programs include diagnostic test procedures, instrument calibration procedures, and extensive instrument logbooks, all of which can facilitate the validation processes of various regulatory agencies. Such complementary functions, although not

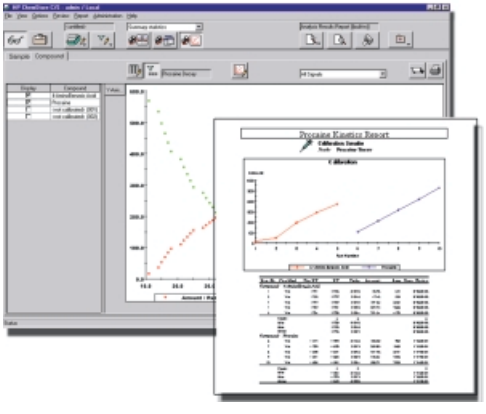


**Figure 76**
**Cross sample reports regression analyses, trend charts and other calculations consolidate sample data, enhancing the overall productivity and efficiency of the laboratory**

directly related to the control of the equipment, are more easily built into a software program than into the equipment itself. In fact, many GLP/GMP features are added to every new version of the software programs sold with HPLC equipment (see figure 77). For example, in some chromatography software, the raw data files can store more than just signal data. A binary check-sum protected file stores instrument parameters (system pressure, temperature, flow, and solvent percent) as well as all aspects of the analytical method, including integration events, calibration settings, and a date-stamped logbook of events as they occurred during the run. Additionally, with spectral libraries, compounds can be identified not only on the basis of their elution profile but also according to their spectral characteristics. Such procedures can be fully automated to reduce analysis time and user interaction.

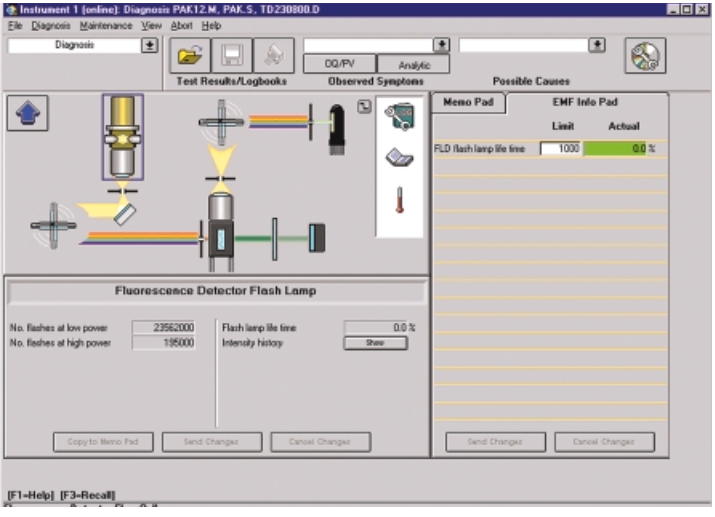



**Figure 77**
**Maintenance and diagnosis screen**

A single PC running the appropriate chromatography software can process data from several detectors simultaneously. This feature is particularly useful in analyses in which sensitivity and selectivity must be optimized to different matrices and concentrations. For example, in the analysis of polynuclear aromatic hydrocarbons, UV absorbance and fluorescence detection are applied in series. The PC displays graphically the chromatographic signals and spectra, enabling detailed interpretation of the data. Software purity algorithms can be used to help determine peak homo-geneity, even for coeluting peaks.

Flexible software programs can report data in both standard and customized formats. For example, some chromatography software can be programmed to yield results on peak purity and identification by spectra or, for more complex analyses, to generate system suitability reports. Any computer-generated report can be printed or stored electronically for inclusion in other documents. PCs are well-suited for the modification of calibration tables and for the reanalysis of integration events and data. The software must record such recalculation procedures so that the analysis can be traced to a particular set of parameters in accordance with GLP/GMP principles.

A computer can automate entire sequences of unattended analyses in which chromatographic conditions differ from run to run. Steps to shut down the HPLC equipment also can be programmed if the software includes features for turning off the pump, thermostatted column compartment, and detector lamp after completion of the sequence. If the HPLC equipment malfunctions, the software reacts to protect the instrumentation, prevent loss of solvents, and avoid unnecessary lamp illumination time. A good software application should be able to turn off the pump, thermostatted column compartment, and detector lamp in the event of a leak or a faulty injection. System suitability tests also can be incorporated in a sequence. When performed on a regular

basis, such tests can validate assumptions about performance of the analytical system and help verify results.

| ✔ | ✘ |
|---|---|
| Enables control of multiple instruments. Additional software can be used for many other tasks. Provides for better data storage and archival. | Requires more bench space for peripherals such as printers or plotters. |

## Local area networks

A laboratory running food analyses frequently requires multiple instruments from multiple instrument vendors for sample analysis. Although the integrators and PC systems described above can evaluate data at analytical instrument stations throughout the laboratory, this data must be collected centrally—over a network, for example—in order to generate a single report for multiple analytical techniques. Local area networks (LANs) offer several advantages in addition to shared data processing (see figure 78). Centralized printing saves bench space and reduces equipment expenses, and centralized file security through a single computer—the server—accelerates data backup. Standard network software and hardware cannot handle data files from diverse analytical instrument vendors. The analytical software therefore should have file conversion utilities based on the Analytical Instrument Association ANDI file format (*.cdf).

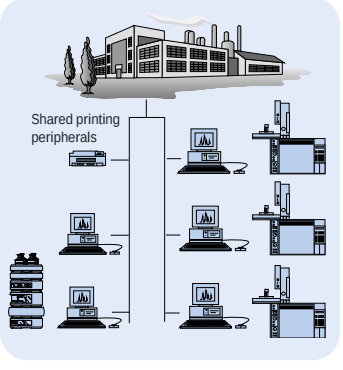



**Figure 78**
**A laboratory LAN — connecting instruments and collating analytical results**

| ✔ | ✘ |
|---|---|
| Integrates multiple techniques and instruments from multiple vendors. Saves bench space and computer processing resources. Access to network utilities such as e-mail. | Data processing features may not match those of dedicated data analysis software applications. |

# Networked data systems

The Agilent ChemStation remote access and data storage modules combine isolated islands of data into a powerful client/server networked information system. Each Agilent ChemStation becomes a network client. It is possible to oversee and control all laboratory operations securely and easily from any computer on the network. The progress of each analysis is monitored to ensure the quality of the results the first time the sample is analyzed. Appropriate action can be taken with the access remote capability from wherever you happen to be if the performance looks suspect. Laboratory data is automatically stored on one centralized and secure server system.

## In brief…

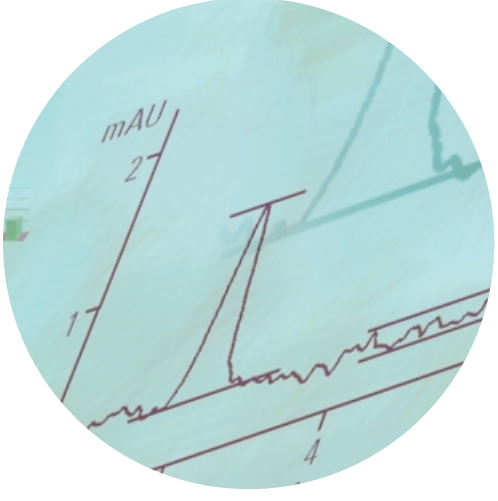
Which data handling technique is most effective and economical for your laboratory depends on several factors:

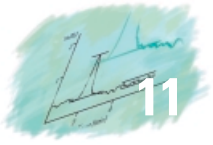
- the size of the laboratory
- the role of the laboratory in the organization
- industrial testing, public safety testing, and so on
- the demands on sample throughput
- the range of analytes under study

For laboratories with few instruments and low sample throughput, integrator systems normally suffice, although a PC may be more appropriate for automated operation of multiple HPLC instruments. A client/server networked data system helps consolidate documentation and validation processes for multiple techniques and instruments from multiple vendors.

*Chapter 11*

# Factors that determine performance in HPLC






**The analysis of food samples places high demands on HPLC equipment, notably in the areas of performance, stability, and reliability. Modern evaluation software enables you to determine the suitability of a particular piece of HPLC equipment for analysis. The factors that influence the outcome of a measurement thus can be identified before results are published to confirm assumptions made during analysis or to draw attention to erroneous data.**

In this chapter we focus on those instrument-related parameters that strongly influence the limit of detection (LOD) and the limit of quantification (LOQ). We also discuss the accuracy, precision, and qualitative information that an HPLC system can provide. Some vendors address the performance of specific instrumentation in technical notes.[43] Such notes include detailed performance test procedures and results for individual modules as well as for complete HPLC systems.

## Limit of detection and limit of quantification

The principle determinant of the LOD in HPLC is the response of the detector to the compound of interest. The response factor thus depends primarily on the choice of detection technique. However, regardless of the quality of the detector, the LOD or LOQ remains a function of peak height. This height can sink if the peak is allowed to disperse within the surrounding liquid in the flow path. All parts of the flow path in front of the detector therefore must be designed to limit broadening and flattening of the response.

A minimum of narrow capillaries between injector and column and from column to detector helps keep dead volume low. With low injection volumes, separation efficiency of the column can be utilized to the maximum, thereby improving peak height. In other words, the lower the column volume, the lower the peak volume eluted. Other factors that influence peak dispersion include pump performance, degassing efficiency, capacity factor (k'), and column particle size. Any improvements can be registered by calculating the S/N of the analyte. Indeed, the noise of the detector should be tested regularly in this way to ensure that performance is maintained. Dead volume of the complete injection system can be determined by first injecting a tracer mobile-phase additive into the flow path with the column disconnected and then recording the time this additive takes to reach the detector at a particular flow rate. The flow cell volume of the detector should be as low as possible, whereas its pathlength should be as long as possible, according to Beer's law.

Maximizing analyte response is not sufficient to ensure good results, however, since the level of background noise from the detector can counter any gains made. In particular, the performance of the pump in combination with certain solvents can increase detector noise level, as described in chapter 7. Degassing is necessary in order to avoid gas

bubbles, which can cause noise or spikes, or oxygen quenching in fluorescence.

High k' values result from higher elution volume or from longer retention time. These values are accompanied by broader peak width and smaller peak height, that is, peaks with longer retention times have poorer S/N. The use of different columns, different mobile phases, and different flow rates can improve S/N. Packing material also directly influences peak dispersion; for example, smaller-sized particles reduce peak dispersion.

## Accuracy and precision

Accuracy is the degree of agreement between test results and true values. It is influenced by the analytical method, the extraction procedure used, and the choice of column or detector. Prior to the adoption of any HPLC method for routine use, the degree of agreement with an established reference method should be determined, or a control run should be performed with a known quantity of spiked sample matrix. In practice, however, the degree of agreement will never reach 100 %. This mismatch can be corrected by calibration with standards of known concentration and, based on these results, by calculating the accurate results from an unknown sample. Inclusion of an external or internal standard calibration procedure ensures accuracy in food analysis. The precision of a method is the degree of agreement among individual test results when an analysis is applied repeatedly to multiple samplings. Precision is measured by injecting a series of standards and then calculating the relative standard deviation of retention times and areas or peak heights. Precision may be measured at three levels: repeatability, intermediate precision, and reproducibility. Repeatability is associated with an analysis performed in one laboratory by one operator using a single piece of equipment over a relatively short time period. Intermediate precision is

the long-term variability of the measurement process for a method performed within one laboratory but on different days. Reproducibility applies to an analysis performed in more than one laboratory. Any HPLC method used in food analysis should be tested for both repeatability and reproducibility.

The precision of a method is strongly influenced by the performance of the HPLC instrumentation. Repeatability of flow rates, gradient formation, and injection volumes can affect precision, as can response stability of the detector, aging of the column, and temperature stability of the column oven. The equipment should be inspected on a regular basis using the test methods recommended by the supplier to ensure reliability, high performance, and good analytical results.

## Qualitative information

HPLC analytes can be identified on the basis of their retention times and either their UV-visible or mass spectra. Compounds, on the other hand, are identified primarily according to the degree of agreement between retention times recorded using calibration standards and those obtained from the sample. Unfortunately, co-eluting peaks can falsify results obtained with samples containing unknowns, especially for food matrices such as meat, vegetables, or beverages. In such cases, samples often can be identified using UV-visible spectral information. A diode array detection system enables online acquisition, and a number of software packages offer automatic evaluation, for example for the analysis of polynuclear aromatic hydrocarbons (PNAs) and pesticides.[41]

References
and Index

# Part Three

# *References*

1. D.N. Heiger, “High Performance Capillary Electrophesis–An Introduction”, *Agilent Primer* 5968-9936E, 2000.
2. CD-ROM ”CE Partner”, *Agilent publication* 5968-9893E
3. CD-ROM ”CE Guidebook”, *Agilent publication* 5968-9892E
4. *Official Methods of Analysis, Food Compositions; Additives, Natural Contaminants*, 15th ed; AOAC: Arlington, VA, 1990, Vol. 2.
5. A.M. Di Pietra, et al., “HPLC analysis of aspartame and saccharin in pharma- ceutical and dietary formulations”, *Chromatographia*, 1990, 30, 215–219.
6. A.G. Huesgen, R. Schuster, “Sensitive analysis of synthetic colors using HPLC and diode-array detection at 190–950 nm”, *Agilent Application Note* 5964-3559E, 1995.
7. A. Herrmann, et al., “Rapid control of vanilla-containing products using HPLC”, *J. Chromatogr*., 1982, 246, 313–316.
8. *Official Methods of Analysis*; W. Horwitz, Ed.; 14th ed.; AOAC: Arlington, VA, 1984; secs 12.018–12.021.
9. H. Malisch, et al., “Determination of residues of chemotherapeutic and antiparasitic drugs in food stuffs of anomaly origin with HPLC and UV-Vis diode-array detection”, *J. Liq. Chromatogr*., 1988, 11 (13), 2801–2827.
10. EC Guideline 86/428 EWG 1985.
11. M.H. Thomas, *J. Assoc. Off. Anal.*; 1989, 72 (4) 564.
12. Farrington et. al., “Food Additives and Contaminants”, 1991, Vol. 8, No. 1, 55-64”.
13. Lebensmittel- und Bedarfsgegenständegesetz, Paragraph 35, Germany.
14. W. Specht, “Organochlor- und Organo-phosphor-Verbindungen sowie stickstoffhaltige sowie andere Pflanzenschutzmittel”, DFG-Methoden sammlung, 1982, 19.
15. ”A new approach to lower limits of detectionand easy spectral analysis”, *Agilent Primer* 5968-9346E, 2000.
16. R. Schuster, “A comparison of pre- and post-column sample treatment for the analysis of glyphosate”, *Agilent Application Note* 5091-3621E, 1992.
17. A.G. Huesgen, R. Schuster, ”Analysis of selected anions with HPLC and electrochemical detection”, *Agilent Application Note* 5091-1815E, 1991.
18. “Determination of triglycerides in vegetable oils”, EC Regulation No. L248, 28ff.
19. L.M. Nollet, *Food Analysis by HPLC* New York, 1992.
20. A.G. Huesgen, R. Schuster, “Analysis of selected vitamins with HPLC and electrochemical detection”, *Agilent Application Note* 5091-3194E, 1992.
21. O. Busto, et al. “Solid phase extraction applied to the determination of biogenic amines in wines by HPLC”, *Chromatographia*, 1994, 38(9/10), 571–578.
22. “Sensitive and reliable amino acid analysis in protein hydrolysates using the Agilent 1100 Series”, *Agilent Technical Note*, 5968-5658E, 2000
23. R. Schuster, “Determination of amino acids in biological, pharmaceutical, plant and food samples by automated precolumn derivatisation and HPLC”, *J. Chromatogr.*, 1988, 431, 271–284.
24. Capillary Liquid Chromatography with the Agilent 1100 Series Modules and Systems for HPLC”, *Agilent Technical Note* 5965-1351E, 1996.

25. R. W. Frei and K. Zech, “Selective sample handling and detection in HPLC”, *J. Chromatogr*. 1988, 39A.
26. D. R. Gere et al., “Bridging the automation gap between sample preparation and analysis: an overview of SFE, GC, GC/MSD and HPLC applied to several types of environmental samples”, *J. Chromatogr. Sci.*, 1993, July.
27. M.A. Schneidermann, et al., *J. Assoc. Off. Anal. Chem.*, 1988, 71, 815.
28. R.Schuster, “A comparison of pre- and postolumn sample treatment for the analysis of glyphosate”, *Agilent Application Note* 5091-3621E, 1992.
29. M. Verzele et al., *J. Am. Soc. Brew. Chem.*, 1981, 39, 67.
30. W.M. Stephen, “Clean-up techniques for pesticides in fatty foods”, *Anal. Chim. Acta*, 1990, 236, 77–82.
31. J.E. Farrow, et al., *Analyst* 102, 752
32. H. Schulenberg-Schell et al., Poster presentation at the 3rd International Capillary Chromatography Conference, Riva del Garda, 1993.
33. S. K. Poole et al., “Sample preparation for chromatographic separations: an overview”, *Anal. Chim. Acta*, 1990, 236, 3–42.
34. R. E. Majors, “Sample preparation perspectives: Automation of solid phase extraction”, *LC-GC Int.* 1993, 6/6.
35. E. R. Brouwer et al., “Determination of polar pollutants in river water using an on-line liquid chromatographic preconcentration system,” *Chromatographia*, 1991, 32, 445.
36. I. McMurrough, et al., *J. Am. Soc. Brew. Chem*., 1988.
37. K. K. Unger, *Handbuch für Anfänger und Praktiker*, 1989, Git Verlag, Germany.
38. W.O. Landen Jr., *J. Assoc. Off. Anal. Chem*., 1985, 68, 183.
39. L. Huber, “Good laboratory practice for HPLC, CE and UV-Visible spectroscopy”, *Agilent Primer,* 5968-6193E, **2000**
40. R. L. Grob, M. A. Kaiser, “Environmental problem solving using gas and liquid chromatography”, *J. Chromatogr.* ,1982, 21.
41. A. G. Huesgen et al., “Polynuclear aromatic hydrocarbons by HPLC”, *Agilent Application Note*, 5091-7260E, 1992.
42. R. Schuster, “A comparison of pre- and postolumn sample treatment for the analysis of glyphosate”, *Agilent Application Note*, 5091-3621E, 1992.
43. H. Godel, “Performance characteristics of the HP 1100 Series modules and systems for HPLC,” *Agilent Technical Note*, 5965-1352E, 1996.

# *Index*

## Numerics


1,4-diaminobutan, 48
1 5-diaminopentane, 48
1-butylamine, 48
1-naphthol, 28
2,2'-dithiobis (5-nitro-pyridine), 108
2,4-dinitrophenyl hydrazine, 108
2-naphthacyl bromide, 108
2-phenylphenol, 58
3-hydroxycarbofuran, 28
3-ketocarbofuran, 28
3-methylbutylamine, 48
9-fluorenylmethyl chloroformate (FMOC), 108


## A


absorption spectrum, 91
accreditation standards, 59
accuracy, 120, 122
acesulfam, 8
acetic acid, 2
acids
  3,3'-thiodipropionic, 4
  acetic, 2
  adipic, 2
  amino, 50
  ascorbic, 4
  benzoic, 5
  butyric, 38
  citric, 2, 3, 43
  fatty, 35, 38, 60,108
  folic, 43
  fumaric, 2
  lactic, 2
  malic, 2
  mercapto-propionic (MPA), 9
  nordihydroguaiaretic, 4
  oxalic, 3
  panthothenic, 43
  phosphoric, 2
  propionic, 2, 5
  sorbic, 2, 6
  succinic, 2
  tartaric, 2
acidulants, 2, 3
additives, III
adipic acid, 2
adsorption chromatography, 59
adulteration, 35
aflatoxins, 21, 22, 23, 60
alcohols, 108
alcoholysis, 45
aldehydes, 108
aldicarb, 28
aldicarb sulfone, 28
aldicarb sulfoxide, 28
alducarb, 28
alkaline hydrolysis, 45
amines, 48
  primary, 108
  secondary, 108
amino acids, V, 50, 99
ammonia, 48
AMPA, 29
amperometric detection, 98
amylamine, 48
animal feed, 18, 21, 22, 44
anions, 33
  inorganic, 32
antibiotics, III
antioxidants, III, 4, 63
apples, 21, 22
artificial sweeteners, III, 8
ascorbic acid, 4
aspartame, 8
atmospheric pressure chemical ionization (APCI), 102 - 104
autoincrement mode, 100
automated injector, 72
autosampler, 72, 109


## B


backflash valve, 67
bacteria, 15
BASIC programming language, 112
beer, 48, 50
Beer's law, 121
benzoic acid, 6
benzothiazuron, 16
BHA butylated hydroxyanisole, 4
BHT butylated hydroxytoluene, 4
biogenic amines, 48
biotin, 43
biphenyl, 84
bisphenol A (BADGE), 24
bitter compounds, 12, 14
bromophenacyl bromide, 38
butocarboxim, 28
butocarboxim sulfone, 28
butocarboxim sulfoxide, 28
butter, 38
butyric acid, 38


## C


calibration
  curves, 113
  settings, 115
  tables, 116
capacity factor, 121
capillary electrophoresis, V
capillary liquid chromatography, 52
carbamates, 26,103,109
carbaryl, 28
carbendazim, 27
carbofuran, 28
carbohydrates, III, 40, 41
Carrez, 7, 14
cell design (electrochemical)
  porous flow-through, 99, 100
  thin-layer, 99, 100
  wall-jet, 98, 100
cellobiose, 39
cereals, 19, 20
cheese, 48
chemical residues, 16
chemotherapeutics, 16
chewing gum, 5
chiral drug, V
chloramphenicol, 15
chlorite, 99
chlorpyripho-ethyl, 27
chromophore, 38, 108
citric acid, 2, 3, 43
cleanup, 54
client/server-based software, 114
cognac, 13
collision induced dissociation (CID), 19
colorants, III, 10
column
  guard, 59, 67
  narrow-bore, 59
  standard-bore, 59
  temperature, 60

compressibility, 79
computer networks, 114
computing equipment, 112
conductivity detector, 86
copper, 99
corn, 41
coulometric detection, 98
counter electrode, 98
cross sample reports, 114

## D

dairy products, 22
dansyl chloride, 49
data
  evaluation, 112
  generation, 112
  storage, 114
dead volume, 59, 71, 121
DEG, 3
degassing, 82
  helium, 83
  ultrasonic, 83
  vacuum, 83, 84
derivatization, 73
  chemical, 62, 108
  postcolumn, 109, 110
  precolumn, 109, 110
detection
  amperometric, 98
  coulometric, 99
detector, 86-105
  conductivity, 86
  diode array, 86, 90, 105
  electrochemical, 86, 87, 98, 105
  electroconductivity, 32
  fluorescence, 87, 95, 105
  mass spectrometer, 86, 88, 101, 105
  refractive index, 86, 87, 104
  response, 88
  thermal energy, 86
  UV, 89, 90
  variable wavelength, 89, 90, 105
deuterium lamp, 10, 90, 92
DG dodecyl gallate, 4
diagnostic test, 114
diethylamine, 48
diode array detector, 87, 91
diquat, 26
direct solvent extraction, 45
drift, 87
drift trigger, 101
drinking water, 26, 33
dual-lamp design, 10
dual-piston mechanism, 78
dyes, V
dynamic range, 88

## E

eggs, 16,17
electrochemical detector, 86, 87, 98, 105
electroconductivity detector, 32
electrospray ionization, 102, 103
elution order, 60
emission, 96, 97
emission grating, 95
enzymatic hydrolysis, 45
essential oils, 12
ethanol, 3
ethanolamine, 48
ethiofencarb, 28
ethiofencarb sulfone, 28
ethiofencarb sulfoxide, 28
ethopabat, 16
ethylamine, 48
excitation, 96, 97
excitation grating, 95
extinction coefficients, 87
extraction
  liquid-liquid, 65
  solid-phase, 63, 65
  supercritical fluid, 64

## F

fats, 35, 37, 38
fatty acids, 35, 38, 60, 108
fertilizers, III
figs, 21, 22
fish, 48
flavors, III, 12
flour, 21, 30
flow
  precision, 79
  ranges, 76
  rates, 76
fluorescence detection, 109
fluorescence detector, 87, 95, 105
fluorescent tag, 109
folic acid, 43
folpet, 27
Food and Drug Administration (FDA), V
food colors, 10
fragmentation, 19
fructose, 40
fruit juices, 6
fruits, 28
fumaric acid, 2
fumonisins, 19
fungi, 15, 21
furazolidone, 15

## G

galactose, 40
gel permeation chromatography (GPC), 27, 66
glassy carbon, 99
GLP/GMP principles, 114
glucose, 40
glycerol, 3
glyphosate, 26, 29
gold, 99
good laboratory practice (GLP), 67
gradient
  elution, 76
  formation, 77
  high pressure, 80
  low-pressure, 78
guard column, 59, 67

## H

halogens, 99
hesperidin, 12, 14
hexylamine, 48
histamine, 48
hormones, III
humulon, 12
hydrazine, 99
hydrogen peroxide, 99
hydrolysis, 38
hydroperoxides, 35, 36

## I

indirect UV-detection, 33
injection volumes, 70
injector
  automated, 72
  manual, 71
  program, 110
inorganic anions, 32
inorganic ions, V
instrument
  calibration, 114
  logbooks, 114
  parameters, 115
integration
  events, 115
parameters, 113
integrators, 112 , 113
interface (MS)
  moving belt, 102
  particle beam, 102
  thermospray, 102
intermediate precision, 122
iodide, 34
ion-exchange chromatography, 10
ion-exchange phases, 58
ionox-100
    4-hydroxymethyl-2,6-di(tert-butyl)
    phenol, 4
ion-pairing reversed-phasechromatography,
    10, 11
ipronidazol, 16
isoabsorbance plot, 91, 92
isobutylamine, 48
isopropylamine, 48

## K

ketones, 108

## L

lactic acid, 2
lactose, 42
LC/MS, 52, 101, 102
LC/MSD, 19, 24
lemonade, 41
light intensity, 91
limit of detection (LOG), 87, 120, 121
limit of quantification (LOQ), 87, 120, 121
linearity, 87, 88
lipids, 35
liquid-liquid extraction, 65
local area network (LAN), 117
long-term variability, 123
luminescence, 95
lupulon, 12

## M

malic acid, 2
maltose, 40
mannitol, 40
manual injector, 71
margarine, 38, 47
mass spectra, 53, 54
mass spectrometer, V, 86, 88, 101, 105
meat, 16, 63
memory effect, 70
mercaptobunzothiazol, 26
mercapto-propionic acid, 9
mercury, 99
mercury-gold, 99
methabenzthiazuron, 26
methanol, 3
methiocarb, 28
methiocarb sulfone, 28
methiocarb sulfoxide, 28
methomyl, 28
methylamine, 48
meticlorpindol, 16
metronidazol, 16
microbial growth, 6
microorganism, 6
microsampling, 70
milk, 16, 21, 22
mixing noise, 81
molecular weight, 66
monochromator, 89
morpholine, 48
moving-belt interface (MS), 102
multichannel integrators, 113
multisignal, 97
multisignal detection, 92
mycotoxins, 21

## N

N-acetyl metabolite, 15
naringenin, 12, 14
narrow-bore column, 59
natural sweeteners, III
Nernst equation, 98
networked data systems, (NDS), 112, 118
nicarbazin, 16
nitrites, 32
nitro compounds, 27
nitrofurans, 16
NOGA nordihydroguaiaretic acid, 4
noise, 87, 122
normalization, 113
normal-phase column, 46, 47
nuts, 21, 22

## O

oat seedlings, 52, 53
ochratoxin A, 21, 22
OG octyl gallate, 4
oils, 35 - 38
one-lamp design, 10
online spectral measurements, 96
o-phthalaldehyde (OPA), 9, 108
orange juice, 14
organic acids, V
oxalic acid, 3
oxamyl, 28
oxidizable sulfur compounds, 108
oxytetracycline, 18

## P

pantothenic acid, 43
paprika, 27
paraquat, 26
partition phases, 58
Patent blue, 10
patuline, 21, 22
p-bromophenacyl bromide, 108
peak
  co-eluting, 123
  dispersion, 121, 122
  elution, 93
  identity, 93, 94
  purity, 93, 94

Peltier control, 60
peptides, 52
performance test, 122
personal computers, 114
pesticides, III, V, 26, 58
petrol ether, 35, 37
PG propyl gallate, 4
PHB-ethyl, 6
PHB-methyl, 6
PHB-propyl, 6
phenethylamine, 48
phenylisocyanate, 108
phenylurea-herbicides, 26
phosphoric acid, 2
photodiode, 89
  array, 91
photomultiplier tube, 97
photoreceptor protein, 52
phytochrome proteins, 52
pistachio nuts, 23
platinum, 99
polycyclic aromatic hydrocarbons, 96
pork muscle, 18
postcolumn derivatization, 28, 29, 109, 110
potassium ferrocyanide, 14
precision, 120, 122
precolumn derivatization, 109, 110
precolumns, 65
preservatives, III, 6, 7, 63
procymidon, 27
propionic acid, 2, 6
propoxur, 28
propylamine, 48
protein precipitation, 14
proteins, 18
protozoa, 16
pulse ripple, 79
pump
  high-pressure gradient, 80
  low-pressure gradient, 78
pumps, 76-84
pungency compounds, 12
pyrazon, 15
pyrrolidine, 48

## Q

qualitative information, 87, 88, 120, 123
quenching effects, 82
Quinolin yellow, 10

## R

raffinose, 40
redox potentials, 98
reference electrode, 98
refractive index detector, 86, 87, 104
regression analysis, 114
reintegration, 113
repeatability, 122
reproducibility, 122
residues, 16, 26
reversed optics, 91
reversed phase, 58
riboflavin 5' phosphate, 43

## S

saccharin, 8, 43
salad, 27
salad dressing, 7
sample
  cleanup, 59
  preparation, 62
  pretreatment, 72, 110
  volume, 72
sampling device, 70
scanning, 96
selected ion mode (SIM), 105
selectivity, 87
separation, 58
sequences, 116
silver, 99
size-exclusion chromatography, 66
slit, 91
smoked sausage, 32
soft drinks, 8
solid-phase extraction, 65
sorbic acid, 2, 6
sorbitol, 39
spectral
  libraries, 115
  resolution, 91
spices, 21, 22, 27
spikes, 122
spreadsheet, 114
standard
  external, 113
  internal, 113
standard-bore column, 59
steam distillation, 64
sterols, 35
strip chart recorders, 112
succinic acid, 2
sucrose, 40
suitability reports, 110
sulfapyridine, 16
sulfite, 99
sulfonamides, 16
sulfur dioxide, 6
supercritical fluid extraction (SFE), IV, 63, 64
surfactants, V
sweeteners, 8
switching valves, 63
system suitability, 116

## T

table salt, 34
tartaric acid, 2
TBHQ mono-tert-butylhydroquinone, 4
TDPA 3,3'-thiodipropionic acid, 4
tetracyclines, 18
tetrahydrofurane, 35
THBP 2,4,5-trihydroxybutyrophenone, 4
thermal
  energy detector, 86
thermal stability, IV
thin-layer chromatography (TLC), 21
thiofanox, 28
thiofanox sulfone, 28
thiofanox sulfoxide, 28
thiosulfate, 99
tocopherols, 4, 45 - 47
tocotrienols, 46
tolerance levels, III
total ion chromatography, 53, 54
toxicity, 8
toxins, 19
tracer, 80, 121
trend charts, 114
triazines, 26
triglycerides, 35 - 39
trypsin, 53
tryptamine, 48
tungsten lamp, 10, 91

## U

ultrasonic bath liquid extraction, 63
UV absorbance, 86
UV detector, 89, 90

## V

validation processes, 113
vanillin, 12, 13
variable volumes, 70
variable wavelength detector, 89, 90
vegetables, 26, 28
vinclozolin, 27
viruses, 15
viscous samples, 60
vitamins, V, 4, 35, 42, 44, 66
  drink, 43
  fat-soluble, 42, 46
  natural, III
  standard, 46
  synthetic, III
  tablets, 42, 43
  water-soluble, 42, 43
vodka, 2

## W

wavelength switching, 96
wine, 2, 7, 48
wool-fiber method, 10
working electrode, 98

## X

xenon flash lamp, 95

## Z

zearalenone, 21, 22
zinc sulfate, 14

# Retention Time Locking: Concepts and Applications

Application

Gas Chromatography

December 1997


## Authors

Vince Giarrocco
Bruce Quimby
Matthew Klee
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808-1610
USA



## Abstract

**The concepts and applications of retention time locking (RTL) are described. RTL simplifies the process of transferring methods from chromatographic instrument to chromatographic instrument, column to column, and detector to detector. The analysis of impurities in styrene according to ASTM D 5135 is used to demonstrate the efficacy of the approach. Using RTL, the retention times matched within an average of 0.16% (0.02–0.03 minute) in constant pressure modes.**



## Key Words

Retention time locking, method validation, styrene analysis, ASTM D 5135, capillary gas chromatography, laboratory productivity


## Introduction

Retention time is the fundamental qualitative measurement of chromatography. Most peak identification is performed by comparing the retention time of the unknown peak with that of a standard. It is much easier to identify peaks and validate methods if there is no variation in the retention time of each analyte.

However, shifts in retention time occur frequently. Routine maintenance procedures such as column trimming alter retention times. In a multi-instrument laboratory running duplicate methods, the retention times for each instrument will differ from each other, even when run under nominally identical conditions. These differences in retention times mean that each instrument must have a separate calibration and integration event table, making it time-consuming to transfer methods from one instrument to another. Differences in retention time also complicate comparison of data between instruments and over time.

Retention time locking (RTL) is the ability to very closely match chromatographic retention times in any Agilent 6890 gas chromatograph (GC) system to those in another 6890 GC system with the same nominal column.

There are several subtle effects that combine to cause retention time differences between similarly configured GC systems. Columns of the same part number can vary slightly in length, diameter, and film thickness.

GC pneumatics can have small variations in the actual inlet pressure applied at a given setpoint. The actual temperature of the GC oven also has minute but real deviations from the indicated value. The sum of these and other effects result in the observed retention time differences between similarly configured GC systems.

The pneumatics and oven temperature control of the 6890 GC have advanced the state of the art in GC hardware accuracy and precision. Agilent's advances in fused silica capillary column technology have resulted in highly reproducible column-to-column retention characteristics. With these advances, retention time precision for a given peak in a single GC setup is usually better than 0.01 minute. However, even with these advances in columns and instrument hardware, the sum of the effects mentioned above can cause retention time differences between identically configured GC systems of as much as 0.4 minute.

It would be impractical to control all of the instrument and column variables to a degree where retention time differences between similarly configured GC systems are removed. There is, however, a means of greatly reducing these differences. By making an adjustment in the inlet pressure, the retention times on a given GC setup can be closely matched to those of a similarly configured GC system. RTL is based on this principle. The process of RTL is to determine what adjustment in inlet pressure is necessary to achieve the desired match in retention times. Agilent RTL software (G2080AA), which integrates into the Agilent GC ChemStation (version A.05.02 or later), provides the tool required to determine the correct inlet pressure quickly and simply.

There are several advantages gained by using RTL in the laboratory. Peak identification becomes easier and more reliable. It is easier to compare data both between instruments and over time. Comparison of data when using different detectors for analyte identification is simplified. Transferring methods from instrument to instrument or laboratory to laboratory is easier because calibration time windows normally will not require readjustment. Validation of system performance is easier. With "locked" GC methods, the development and use of retention time data bases for unknown identification is much more straightforward.

To maintain a locked method, RTL should be performed whenever:

- The column is changed or trimmed
- The method is installed on a new instrument
- A detector of different outlet pressure is used
- System performance is validated
- Troubleshooting chromatographic problems

To lock a given method for the first-time or for the reasons below, one must first develop a retention time versus pressure (RT vs. P) calibration.

Even when using columns with the same part number (same id, stationary phase type, phase ratio, and same nominal length), separate/different locking calibration curves are needed when using:

- Systems with different column outlet pressures (FID/atmospheric, MSD/vacuum, AED/elevated)
- Columns differing from the "nominal" length by more than 15% (e.g., due to trimming)
- Systems where the predicted locking pressure falls outside the range of the current calibration

A specific solute (usually one found in the normal method calibration standard) must be chosen and then used for both developing the locking calibration and locking all future systems. The solute, or target peak, should be easily identifiable, symmetrical, and should elute in the most critical part of the chromatogram. Solutes that are very polar or subject to degradation should be avoided.

Once the target solute has been chosen and all other chromatographic parameters of the method have been determined, five calibration runs are performed. The runs are made at conditions identical to the nominal method except that four of the runs are made at different pressures. The pressures used are typically:

- Target pressure – 20%
- Target pressure – 10%
- Target pressure (nominal method pressure)
- Target pressure + 10%
- Target pressure + 20%

The retention time of the target compound is determined for each run. The resulting five pairs of inlet pressures and corresponding retention times are entered into the ChemStation software to generate an RTL calibration file.

Figure 1 shows the dialog box used to enter the calibration data. After the data is entered, a plot is displayed, as shown in figure 2. The maximum departure of the fitted curve from the data is given for both time and pressure. If the fit is acceptable, the retention time versus pressure calibration is stored and becomes part of the GC

method. This calibration need only be generated once. Subsequent users of the method can use this calibration when running the method on a similar instrument setup, regardless of location.

To relock a system or lock a new one:

1. Set up the method conditions and run a standard containing the target compound.
2. Enter the actual retention time of the target compound into the "(Re)Lock current method" dialog box (see figure 3).
3. Update the 6890 method with the new calculated pressure, and save the method.
4. Validate the retention time lock by injecting the standard at the new pressure, and compare the retention time obtained to the desired retention time.
5. Repeat steps 2 to 4, if necessary.

## A Note on Constant Flow versus Constant Pressure Modes of EPC Operation

Many GC chromatographers prefer to use the "constant flow mode" of EPC operation. In this mode, inlet pressure increases automatically to maintain constant outlet flow rate as the oven temperature increases during the run. Constant flow mode reduces run time and ensures that flow-sensitive detectors see a constant column effluent flow.

The "constant pressure" mode of EPC operation is also popular. In this mode, the pressure remains constant during the run (outlet flow will decrease as temperature increases). For those wishing to reduce run time in constant pressure mode, a higher pressure can be chosen. For

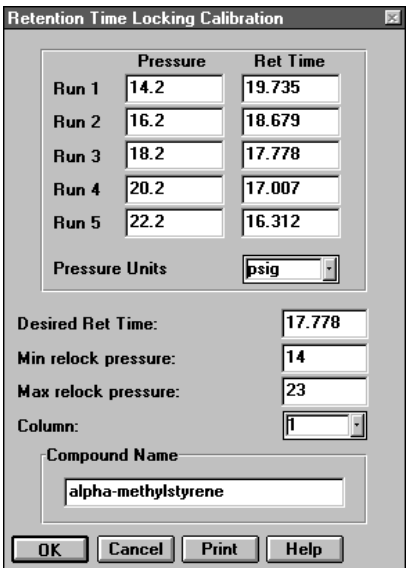



**Figure 1. Dialog box used for entering retention time locking calibration data**

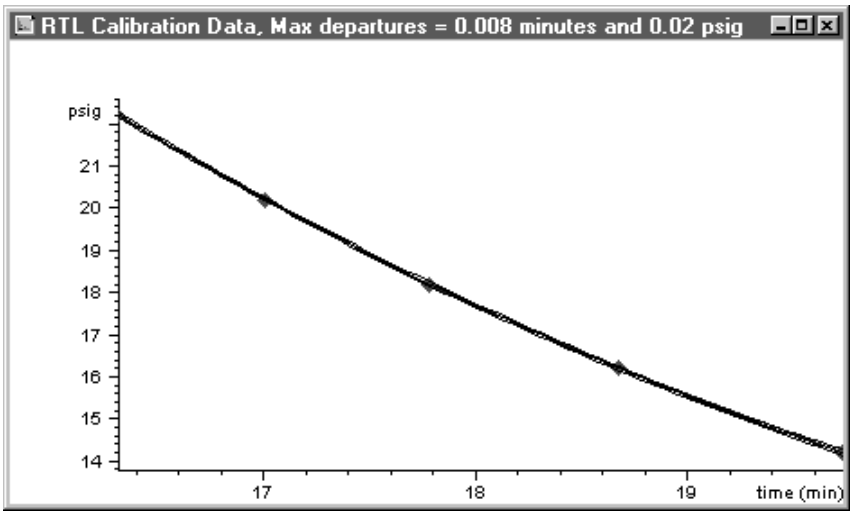



**Figure 2. Plot of calibration data as displayed by RTL software**

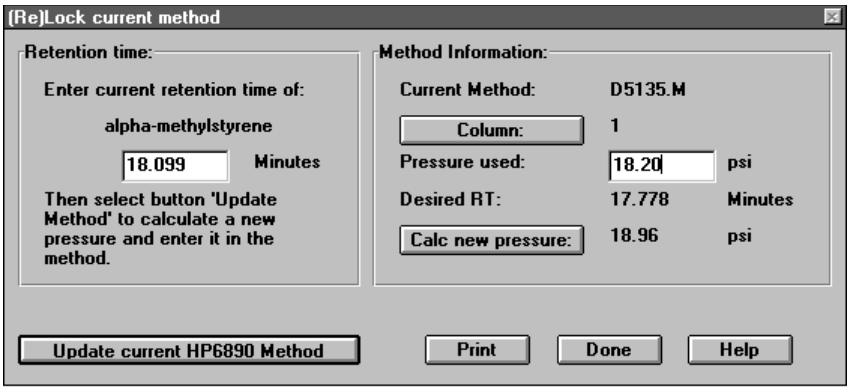



**Figure 3. Dialog box used to calculate locking pressure and update the 6890 method**

flow-sensitive detectors, one can set "constant column flow + makeup" via the 6890 keyboard or ChemStation. In this mode, the makeup flow is increased as the column flow decreases to keep the sum of the two constant.

The underlying theory of RTL predicts that constant pressure mode of EPC provides the closest matching of retention times. If one desires to compare data from systems with very different configurations, such as GC/FID to GC/MSD, it is best to use constant pressure mode. As can be seen from the styrene analysis data herein, retention time matching between systems of the same configuration (GC/FID, in this case) is still quite good in the constant flow mode.

This application note shows the use of RTL to lock retention times between multiple chromatographic instruments, columns, and detector types and demonstrates RTL in both constant flow and constant pressure modes.

## Experimental

Two 6890 Series GC systems were used. Each system was equipped with:

- Electronic pneumatics control (EPC)
- Split/splitless inlet (250 °C, He carrier gas, split 80:1)
- Automatic liquid sampler
- GC ChemStation (version A.05.02)
- Flame ionization detector (FID)
- 60 m × 0.32 mm, 0.5 mm HP-INNOWax column (part no. 19091N-216)
- Temperature program: 80 °C (9 min), 5 °C/min to 150 °C

The inlet pressures/flows used are indicated with each chromatogram.

A third 6890 Series GC was also used. This system was equipped with an Agilent 5973 mass selective detector (MSD) and was used for peak identification. The GC-MSD chromatographic parameters used were the same as the GC systems noted above except for the inlet pressures as indicated.

## Results and Discussion

### GC-FID to GC-FID Locking

Figure 4 shows the original chromatogram (GC system 1) obtained from running a styrene sample under the conditions specified in ASTM D 5135.[1] Many of the typical impurities found in styrene are found here. The phenylacetylene peak represents about 60 ppm. The peaks are identified in table 1.

The sample was then run at four other pressures to collect the five data pairs for RTL calibration. Because this method was run in constant flow mode, the pressures entered into the RTL software were the initial pressures. The α-methylstyrene peak (peak 10) was chosen as the target compound. The calibration data are shown in figure 1.

The method conditions and RTL calibration were then moved to GC system 2, a different GC and column. The sample was run at the original method inlet pressure of 18.2 psi. The chromatogram obtained using this scouting run is overlaid on the original chromatogram in figure 5. The retention times shifted about 0.3 minute on the second GC. This is a typical result obtained when trying to replicate an analysis on a second instrument or with a second column.

The retention time of α-methylstyrene was entered into the RTL software

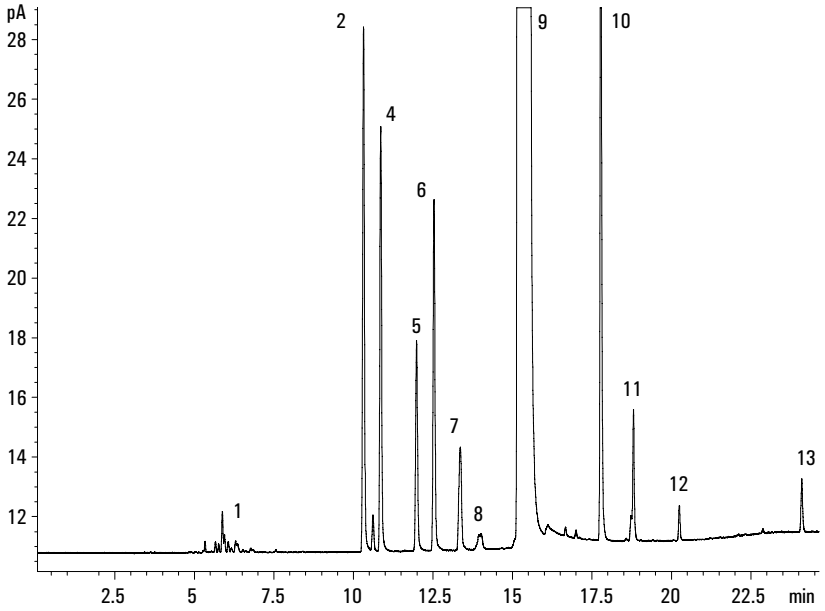



**Figure 4. Styrene sample run on GC system 1 at 18.2 psi initial pressure, constant flow mode**

dialog box on GC system 2, as shown in figure 3. The RTL software indicated the initial pressure should be modified from 18.2 psi to 18.96 psi. The new initial pressure was entered into the method and saved.

Figure 6 compares the chromatograms obtained from the original run and after locking retention times using the α-methylstyrene. Table 2 compares the retention times before and after using this approach. The retention times are now closely matched.

## GC-FID to GC-MSD Locking

A second experiment was conducted to lock the original method from GC system 1 to the GC-MSD. This is useful for identification of unknown impurities that show up in the FID chromatogram. For example, there is a shoulder evident on the front side of the phenylacetylene peak in figure 4. It would simplify locating the impurity in the GC-MSD data if the retention times closely matched that of the GC-FID.

Because constant pressure mode is preferred when comparing data from FID and MSD systems, constant pressure mode was chosen, and the styrene sample was re-run on GC system 1 at 18.2 psi for reference.

The next step was to determine the chromatographic conditions to be used on the GC-MSD. The Agilent method translation software tool was used to calculate the conditions necessary to have the peaks elute in the identical order on the two systems.[2,3] Because the retention times need to match, the dead time and temperature program used for running the GC-MSD must be the same as the GC

**Table 1. Peak Identities for Figure 4**

| Peak # | Name | Peak # | Name |
|---|---|---|---|
| 1 | Nonaromatics | 8 | p/m-Ethyltoluene |
| 2 | Ethylbenzene | 9 | Styrene |
| 3 | p-Xylene | 10 | α-Methylstyrene |
| 4 | m-Xylene | 11 | Phenylacetylene |
| 5 | i-Propylbenzene | 12 | β-Methylstyrene |
| 6 | o-Xylene | 13 | Benzaldehyde |
| 7 | n-Propylbenzene | | |

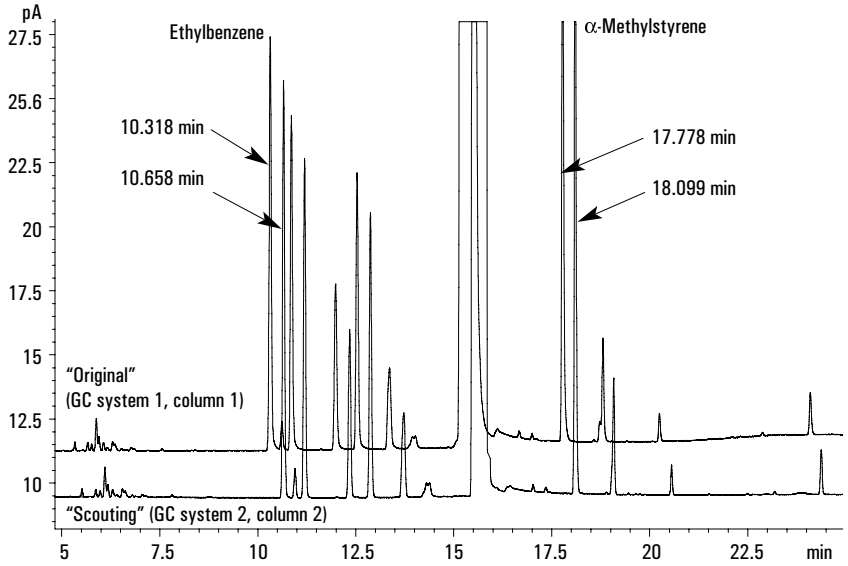



**Figure 5. Comparison of original chromatogram on GC system 1 with GC system 2 before retention time locking**

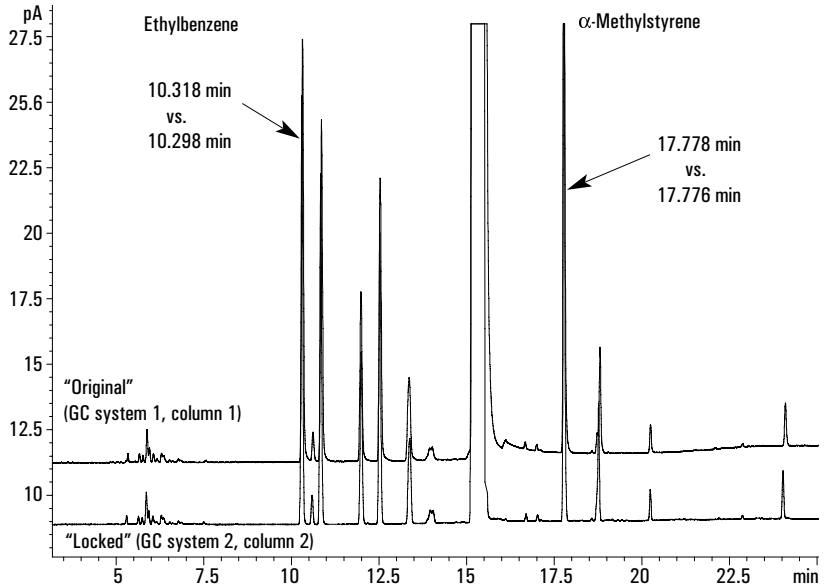



**Figure 6. Comparison of original chromatogram on GC system 1 with GC System 2 after retention time locking**

method. The pressure used, however, will be different due to the difference in column outlet pressure. The GC-MSD inlet pressure is calculated using the "none" mode of the method translation software (figure 7). In this mode, the holdup time between the two columns was forced to be identical to the GC-FID. This gives a speed gain of 1. The pressure calculated for use on the GC-MSD was 8.44 psi. Note that this calculated pressure is only the nominal pressure required to get similar retention times, not the exact locking pressure.

A different RTL calibration is required for GC-MSD because the outlet pressure is vacuum, and that of the FID is atmospheric pressure. Five runs were made on the GC-MSD system bracketing the 8.44 psi nominal method pressure. Because the GC-MSD used in this study was not equipped with RTL software, a dummy method was created in GC system 1 and the GC-MSD RTL calibration data was entered into it. A scouting run of the Styrene sample was made on the GC-MSD, and the α-methylstyrene retention time was used for locking. The locking inlet pressure calculated with the dummy method was 7.9 psi and was entered into the GC-MSD.

Figure 8 shows the resulting matched chromatograms from the GC-FID and GC-MSD. As seen in table 3, the retention times are now closely matched within 0.02 minute.

Figure 9 shows the MSD first choice of library search result of the impurity that created the shoulder on the front side of the Phenylacetylene peak. RTL ensured that this shoulder remained separated on the MSD system and eluted at the same time for easy comparison to the FID results.

**Table 2. GC-FID Retention Times Before and After Locking for Styrene Impurities (Constant Flow Conditions). Chromatograms Shown in Figures 4, 5, and 6.**

| Component | Original Run GC 1/Column 1 18.2 psi | GC2–GC1 Before RTL | Scouting Run GC 2/Column 2 18.2 psi | GC2–GC1 After RTL | Locking Run GC 2/Column 2 19.0 psi |
|---|---|---|---|---|---|
| Ethylbenzene | 10.318 | 0.340 | 10.658 | –0.020 | 10.298 |
| p-Xylene | 10.616 | 0.333 | 10.949 | –0.026 | 10.590 |
| m-Xylene | 10.858 | 0.337 | 11.195 | –0.022 | 10.836 |
| i-Propylbenzene | 11.985 | 0.359 | 12.344 | +0.005 | 11.990 |
| o-Xylene | 12.533 | 0.345 | 12.878 | –0.012 | 12.521 |
| n-Propylbenzene | 13..360 | 0.364 | 13.724 | –0.016 | 13.376 |
| α-Methylstyrene* | 17.778 | 0.321 | 18.099 | –0.002 | 17.776 |
| Phenylacetylene | 18.806 | 0.275 | 19.081 | –0.040 | 18.766 |
| β-Methylstyrene | 20.248 | 0.310 | 20.558 | –0.006 | 20.242 |
| Benzaldehyde | 24.097 | 0.279 | 24.376 | –0.069 | 24.028 |
| Average Δ | | 0.326 | | 0.028 | |

* Used in locking calculation

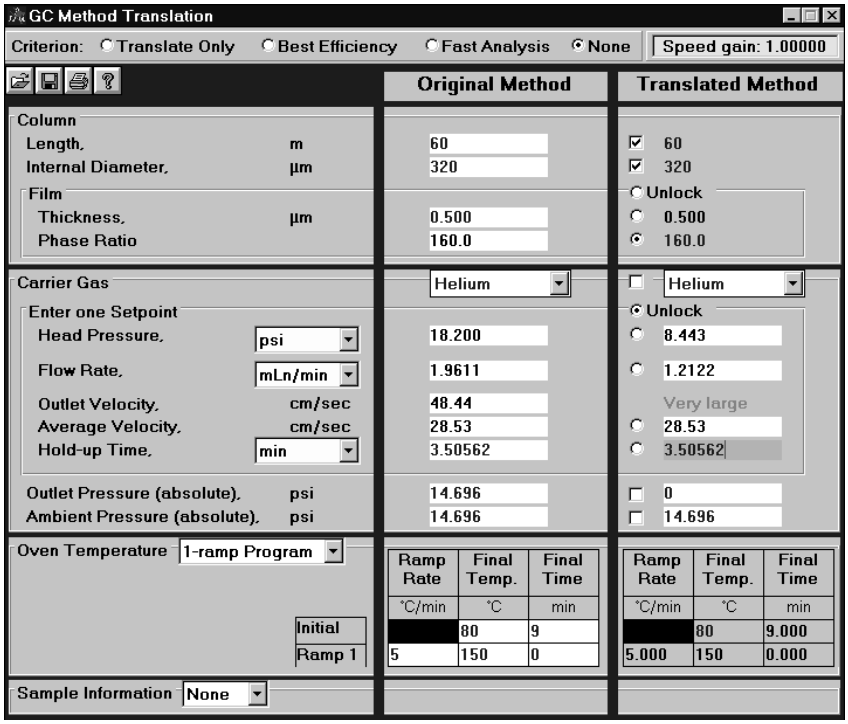

GC Method Translation
Criterion: Translate Only Best Efficiency Fast Analysis None
Speed gain: 1.00000
Original Method
Translated Method
Column
Length, m 60 60
Internal Diameter, µm 320 320
Film
Thickness, µm 0.500 Unlock 0.500
Phase Ratio 160.0 160.0
Carrier Gas Helium Helium
Enter one Setpoint
Head Pressure, psi 18.200 Unlock 8.443
Flow Rate, mLn/min 1.9611 1.2122
Outlet Velocity, cm/sec 48.44 Very large
Average Velocity, cm/sec 28.53 28.53
Hold-up Time, min 3.50562 3.50562
Outlet Pressure (absolute), psi 14.696 0
Ambient Pressure (absolute), psi 14.696 14.696
Oven Temperature 1-ramp Program
Ramp Rate °C/min Final Temp. °C Final Time min
Initial 80 9 80 9.000
Ramp 1 5 150 0 5.000 150 0.000
Sample Information None

**Figure 7. Method translation software provides scaled conditions for GC systems with different configurations**

## Conclusions

Retention time locking facilitates replicating results from instrument to instrument, from column to column, and from detector to detector by locking retention times. The retention times of a styrene sample analyzed according to ASTM D 5135 matched to within 0.06 minute after locking.

## References


1. ASTM D 5135-95, "Analyses of Styrene by Capillary Gas Chromatography," Annual Book of Standards, Volume 06.04, ASTM, 100 Bar Harbor Drive, West Conshohocken, PA 19428 USA.
2. M. Klee and V. Giarrocco, "Predictable Translation of Capillary GC Methods for Fast GC" Agilent Technologies, Inc., Application Note 228-373, Publication 5965-7673E, March 1997.
3. GC Pressure/Flow Calculator for Windows, Version 2.0 and Method Translation Tool Version 2.0. Available at http://www.chem.agilent.com/servsup/usersoft/main.html.

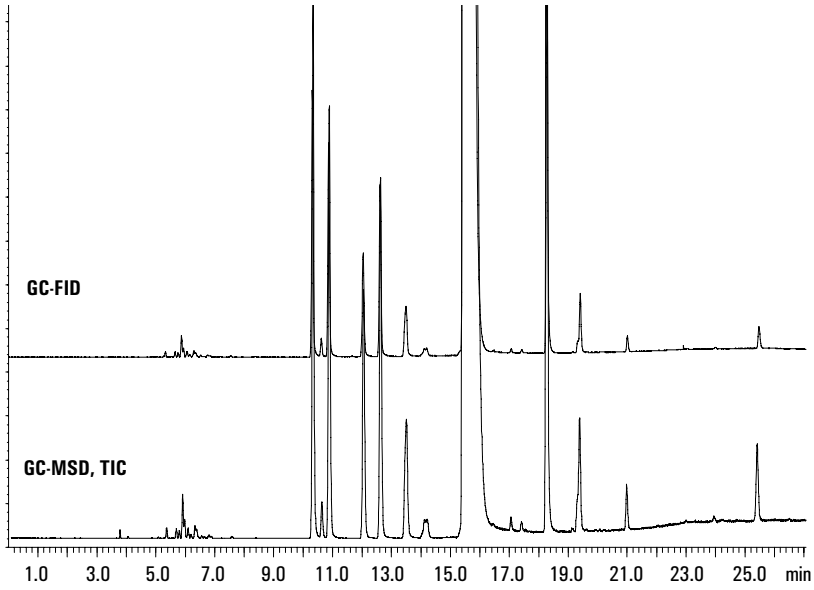




**Figure 8. Comparison of chromatogram on GC system 1 with GC-MSD system after retention time locking, Constant Pressure Mode**

**Table 3. GC-FID vs. GC-MSD, Method Translated then Locked—Retention Times (Constant Pressure Conditions)**

| Component | GC-FID Original 18.2 psi | GC-MSD 7.9 psi | | RT Difference min |
|---|---|---|---|---|
| Ethylbenzene | 10.315 | 10.338 | | 0.023 |
| p-Xylene | 10.620 | 10.642 | | 0.022 |
| m-Xylene | 10.869 | 10.890 | | 0.021 |
| i-Propylbenzene | 12.038 | 12.053 | | 0.015 |
| o-Xylene | 12.613 | 12.630 | | 0.017 |
| n-Propylbenzene | 13.492 | 13.508 | | 0.016 |
| a-Methylstyrene* | 18.276 | 18.267 | | –0.009 |
| Phenylacetylene | 19.406 | 19.389 | | –0.017 |
| b-Methylstyrene | 21.008 | 20.987 | | –0.011 |
| Benzaldehyde | 25.475 | 25.415 | | –0.060 |
| | | | Average | 0.021 |

* Used in locking calculation

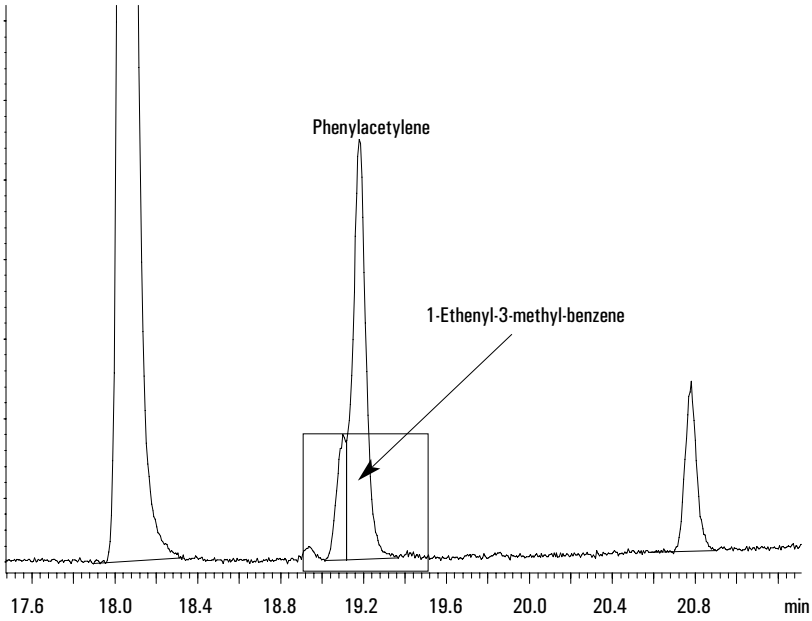



**Figure 9. GC-MSD identification of impurity in shoulder of phenylacetylene peak**

Agilent shall not be liable for errors contained herein or for incidental or consequential damages in connection with the furnishing, performance, or use of this material.

Information, descriptions, and specifications in this publication are subject to change without notice.

Copyright © 2000
Agilent Technologies, Inc.

Printed in the USA 3/2000
5966-2469E

**Agilent Technologies**
Innovating the HP Way

# Large Volume Injection for Gas Chromatography Using a PTV Inlet

Application

Gas Chromatography

March 1997


## Authors

Bill Wilson, Philip L. Wylie,
and Matthew S. Klee
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808-1610
USA



## Abstract

**Reduced sample detection limits is a continuing goal in gas chromatography. Large sample injection volume is one possible approach. New inlets and injection techniques supporting large volume injection (LVI) have been developed in recent years. This paper discusses the uses and limitations of LVI, describes LVI with a programmable temperature vaporizer (PTV) inlet, and reviews the results of LVI analysis of pesticides and straight-chain hydrocarbons.**



## Key Words

Large volume injection, LVI, programmed temperature vaporizer, PTV, gas chromatography, GC, pesticide analysis, hydrocarbon analysis


## Introduction

The demand for lower detection limits is important in environmental, pharmaceutical, food analysis, and other gas chromatography (GC) applications. This demand has driven instrument manufacturers to provide more sensitive instrumentation and procedures, which has prompted regulators to reduce allowable limits, and so on in a never-ending cycle. Improvements in sample handling, sample injection techniques, and detectors have all contributed to the ability to measure compounds at decreasing levels.

Concentrating samples is an established approach for increasing method sensitivity. For many environmental analysis, this involves extraction followed by solvent evaporation, which generates large volumes of waste solvent and increases sample preparation time significantly. Recent advances, such as supercritical fluid extration (SFE), solid-phase extraction (SPE), solid-phase microextraction (SPME), and pressurized fluid extraction, are making inroads on liquid/liquid extractions, but these still involve additional sample preparation time.

Detection limits can be reduced by lowering system background and interferences. This can be done through sample cleanup, such as florisil column chromatography, SPE, and SPME, and by using selective detectors that do not respond to the background. How-ever, the cleanup requires time, and the need for reduced limits has surpassed the sensitivity of even the best detectors.

Large volume injection (LVI) is another approach to lower detection limits. The typical injection volume for capillary column analysis is 0.5 to 2 µL. Agilent 6890 Series and 5890 gas chromatographs (GCs) allow approximately two times the normal injection volume (up to 5 µL, depending on the solvent) using "pulsed" splitless injection. Injecting still larger volumes with standard techniques can lead to contamination of the system, irreproducible results, and loss of sample. With the new LVI technique, good chromatography can be obtained with injection volumes of 5 to 500 µL or more.

Table 1 summarizes several common ways to lower detection limits.

**Table 1. Approaches to Lowering Detection Limits**

| Concentrate the Sample | Transfer More Sample into the Column | Use More Sensitive or Selective Detectors | Decrease System Noise |
|---|---|---|---|
| Extraction followed by evaporation of solvent | Cool on-column (COC) injection | Electron capture detector (ECD) | Selective detectors |
| Solid-phase extraction (SPE) | Splitless injection | Electrolytic conductivity detector (ELCD) | Sample cleanup to reduce interferences |
| Solid-phase micro extraction (SPME) | Large volume injection (LVI) | Atomic emission detector (AED) | Use headspace or purge and trap sampling |
| Headspace sampling | | Selected-ion monitoring mass spectrometry (SIM-MS) | Decrease column bleed |
| Purge and trap (P&T) sampling | | Nitrogen-phosphorus detector (NPD) | |
| Large volume injection (LVI) | | Flame photometric detector (FPD) | |

In LVI, a large volume of sample is injected. The bulk of the solvent is evaporated before transfer of the sample to the analytical column and the start of the analytical sepa-ration. There are two primary techniques to eliminate solvent: PTV and cool on-column injection with solvent vapor exit (COC-SVE). COC-SVE is most appropriate for clean samples with volatile (early-eluting) compo-nents such as extracts of drinking water. The COC-SVE technique is discussed elsewhere.[1]

## PTV

LVI with PTV is ideal for trace analysis of later eluting solutes (boiling points approximately 100°C higher than the solvent) and for dirty samples. Typical injection volumes for solvent elimination PTV are 25 to 100 µL. Injection volumes up to 1 mL have been demonstrated.[2] The large sample volumes are injected by manual injection, by multiple sample injections from a standard automatic sampler, or by LVI with a variable-speed injector.

An automatic injector is recommended for maximum reproducibility. A standard automatic sampler making repeat injections is more cost effective than purchasing a variable-speed sampler and requires less solvent for syringe cleaning.

The Agilent 6890 Series GC uses a standard automatic sampler with syringe sizes up to 50 µL. A 50-µL syringe can inject up to 25 µL. Multiple injections can be used with the PTV inlet when even larger volumes are required. With the 6890 GC system, delay between injections can be controlled as well as the number of injections or the total injection volume. Injection parameters are set through the Agilent ChemStation.

A problem with multiple injections is the increased number of punctures of the GC inlet and vial septa. This reduces septum life and increases the possibility of contamination of sample and inlet. Using a "septumless head" for the inlet can eliminate the inlet problems. Figure 2A shows septum cap extract that contaminated the sample after the vial was pierced 40 times during several multiple-injection experiments. 100% Teflon septa minimize sample contamina-tion such as this, but once punctured, Teflon septa do not reseal.

The PTV inlet can be considered a temperature-programmable split/ splitless inlet with the same basic configuration. While it can be used hot for split and splitless applications, this is not recommended because the volume of vaporized solvent may exceed the low internal volume of the PTV inlet. PTV is ideal for cold split or splitless applications, avoiding most of the problems associated with hot inlets such as sample discrimination, liner overload, and sample decomposition.

For large volume injections, the PTV is used in a "solvent vent" or "solvent elimination" mode. Sample is introduced into the inlet with the inlet temperature near the boiling point of the solvent and with a relatively high split ratio. The solvent (and low-boiling solutes) is vented while the higher boiling solutes (more than about 100°C above the solvent boiling point) remain and are concentrated in the inlet. After a preset time, the split vent is closed and the inlet temperature increased to transfer the solutes and any residual solvent to the column for separation.

Because the sample is evaporated from the inlet, nonvolatile sample components and degradation products remain behind in the inlet, minimizing column contamination. There is evidence that inlet contamination in PTVs influences subsequent injections less than in hot inlets. If contamination becomes an issue, the inlet liner is easily changed. Thus, PTV is a better choice for dirty samples than cool-on-column and split/ splitless inlet.

For a PTV inlet to work well, inlet temperature must be programmed independently from the column oven. The 6890 provides this function. For example, the inlet can be heated with the split flow off to transfer the sample to the column before the oven temperature program begins. After sample transfer, the inlet can be heated further to bake off contaminants with a high split flow to minimize inlet contamination.

LVI by PTV is not a good choice when the target compounds include highly volatile species because these low-boiling compounds are vented along with the solvent. The lowest boiling target compound should boil at least 100°C above the solvent to have a reasonable chance of success.

Table 2 lists the advantages and disadvantages of LVI by solvent elimination PTV.

**Table 2. Advantages and Disadvantages of LVI by Solvent Elimination PTV**

| Advantages | Disadvantages |
|---|---|
| Most flexible LVI technique | Loss of volatile sample components |
| Good for late-eluting compounds such as pesticides, PAHs, etc | Possibility of sample decomposition(although less than with split/splitless) |
| Inlet protects column so one can use dirty samples | More difficult to use than conventional inlets like split/splitless |

## Experimental

A 6890 GC with electronic pneumatics control (EPC) was used. A G1916A automatic liquid sampler (ALS) with a G1513A controller performed sample injection. An Agilent ChemStation (version A.04.02) controlled the instruments and acquired and processed data.

Table 3 lists the hardware and software revisions that support multiple injections, postdwell time, and the slow plunger mode required for LVI-PTV.

Experimental conditions for the GC methods are given with the chromatograms.

**Table 3. Software, Hardware, and Firmware Versions that Support LVI-PTV**

| Item | Software/Firmware |
|---|---|
| ChemStation software | A.04.02 or higher |
| G1513A injector | A.09.10 |
| G1512A ALS | A.01.08 |
| 6890 GC | A.02.01 or higher |

## Results and Discussion

### Multiple Injections

Multiple injections are a straight-forward and reliable way to introduce large sample volumes into the inlet. Figure 1 shows the linearity obtained from two sets of multiple injections using a standard G1513 ALS.

Depending on solvent type and injection volume, liquid sample may run down the liner and enter the column. If this occurs, the column may overload with solvent causing catastrophic peak splitting and possible damage to the stationary phase. To minimize this possibility, a packed liner should be used with multiple injections of more than 5 µL.

In addition to preventing solvent from flowing into the column, glass wool or other packing provides a surface to retain a film of solvent which, in turn, helps retain early-eluting compounds. Figure 2A shows loss of analytes eluting before C18. Figure 2B, with glass wool packing in the liner, shows complete recovery down to C14.

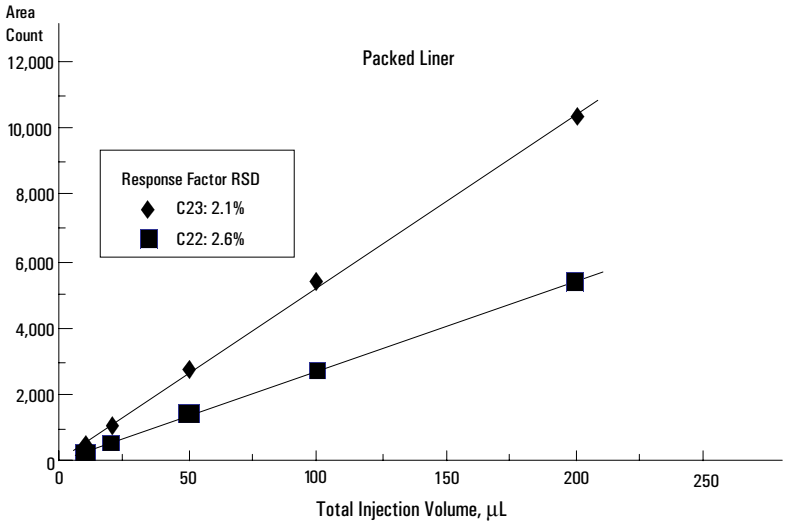



**Figure 1. Linearity of multiple injections**

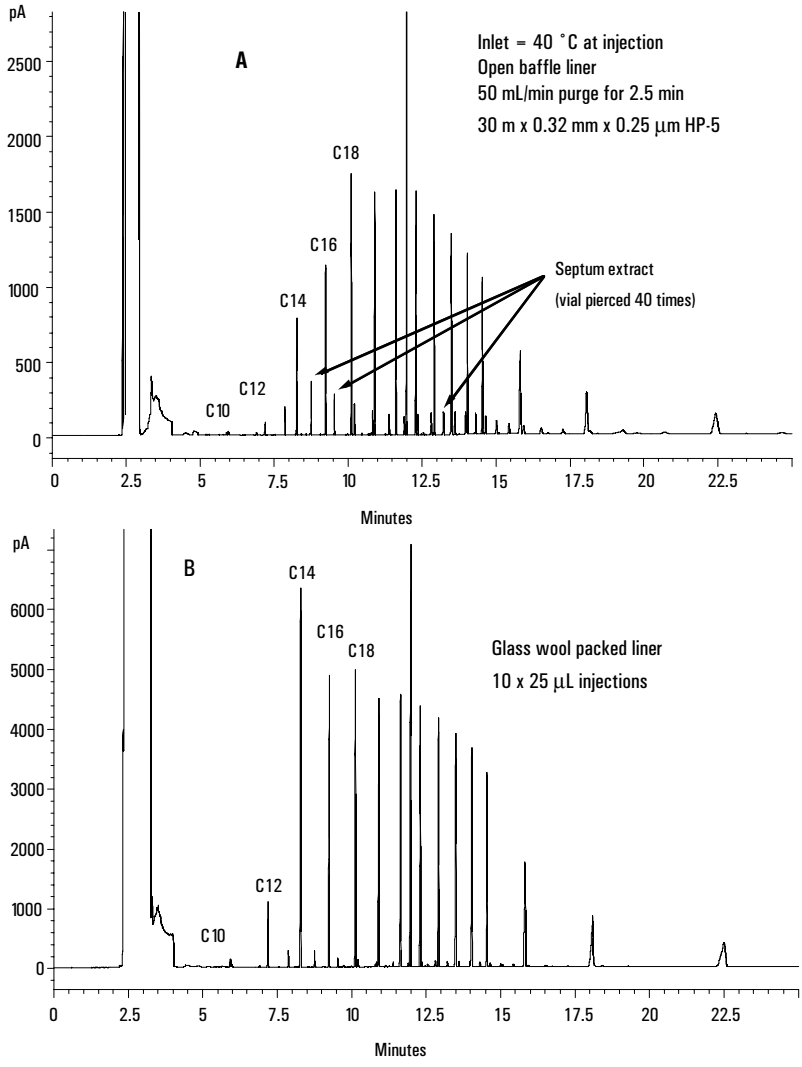



**Figure 2. Comparison of packed and open baffle liners using PTV sample: C10-C44 in Hexane**

## PTV

Figure 3 is a chromatogram of an LVI of pesticides using a PTV inlet. By injecting 25 µL divided into five injections, good response is obtained from a 0.01-ppm mixture

COC-SVE is usually the preferred technique for samples with low-boiling components, because volatile compounds evaporate with the solvent (as shown in Figure 2A) with PTV. However, for volatile samples that are too dirty for COC-SVE sampling, the PTV inlet can be cryocooled below ambient temperature resulting in much better recovery of the low boilers. Figure 4 compares the recovery of C10 using PTV with the inlet temperature at 40°C and -10°C during the injection step. There is 100% recovery of C10 with cryocooling. Much less solvent was eliminated at the lower temperature, which helped retain the early-eluting compounds.

It is important to determine carefully the best injection parameters for LVI methods. Figure 5 shows the improvement in peak shape obtained for a sample containing pesticides when the initial PTV temperature, vent flow, and injection delay are optimized. Inlet temperature and vent flow influence the speed and extent of solvent removal. The chromatogram in figure 5B indicates that insufficient solvent was vented, thereby increasing the chance of carry-over,degrading peak shape, andincreasing ghost peaks.

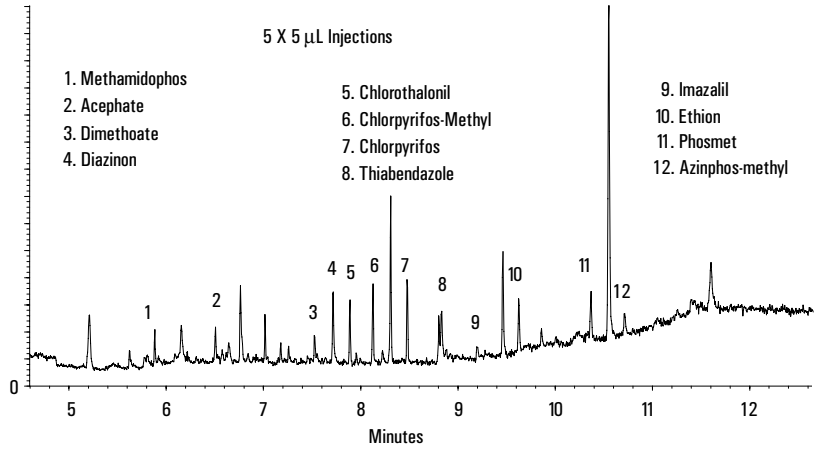



**Figure 3. LVI with solvent elimination PTV**
**Pesticides: 0.01 ppm; PTV conditions: vent flow, 300 mL/min;vent pressure: 0 until 1 min; purge flow: 50 mL/min at 3.50 min; gassaver on at 4.70 min; PTV initial temperature: 20 °C; PTV initial time:1.1 min; PTV rate: 700 °C/min; PTV final temperature: 300 °C; injection delay: 0.00 min; column: 30 m x 0.25 mm x 0.25 µm HP-5MS**

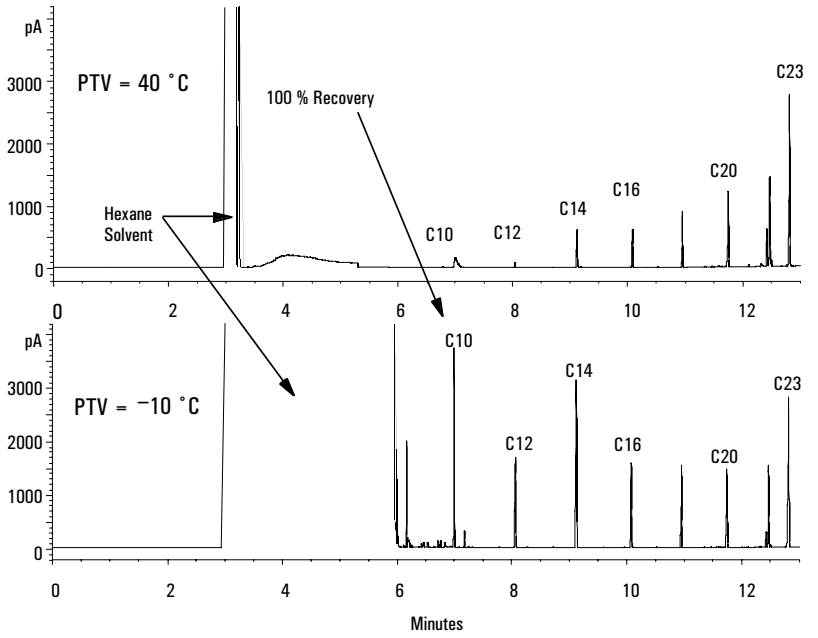



**Figure 4. PTV with cryocooled inlet**

## Conclusion

GC using LVI is useful for lowering detection limits. It has broad applicability for applications that require more sensitivity than can be obtained with standard injection volumes. PTV inlets are appropriate for LVI of late-eluting samples, for dirty samples, and for any cold split/splitless injection. Total automation of temperature, pressure, and flow simplifies PTV use. LVI with solvent elimination PTV requires careful method development for maximum accuracy and reproducibility.

## References


1. B. Wilson, et al., "Large Volume Injection for Gas Chromatography UsingCOC-SVE," Agilent Technologies, Application Note 228-377, Publication Number (23)5965-7923E, March 1997.
2. J. Staniewski and J. Rijks, HRC, 16(1993)182.

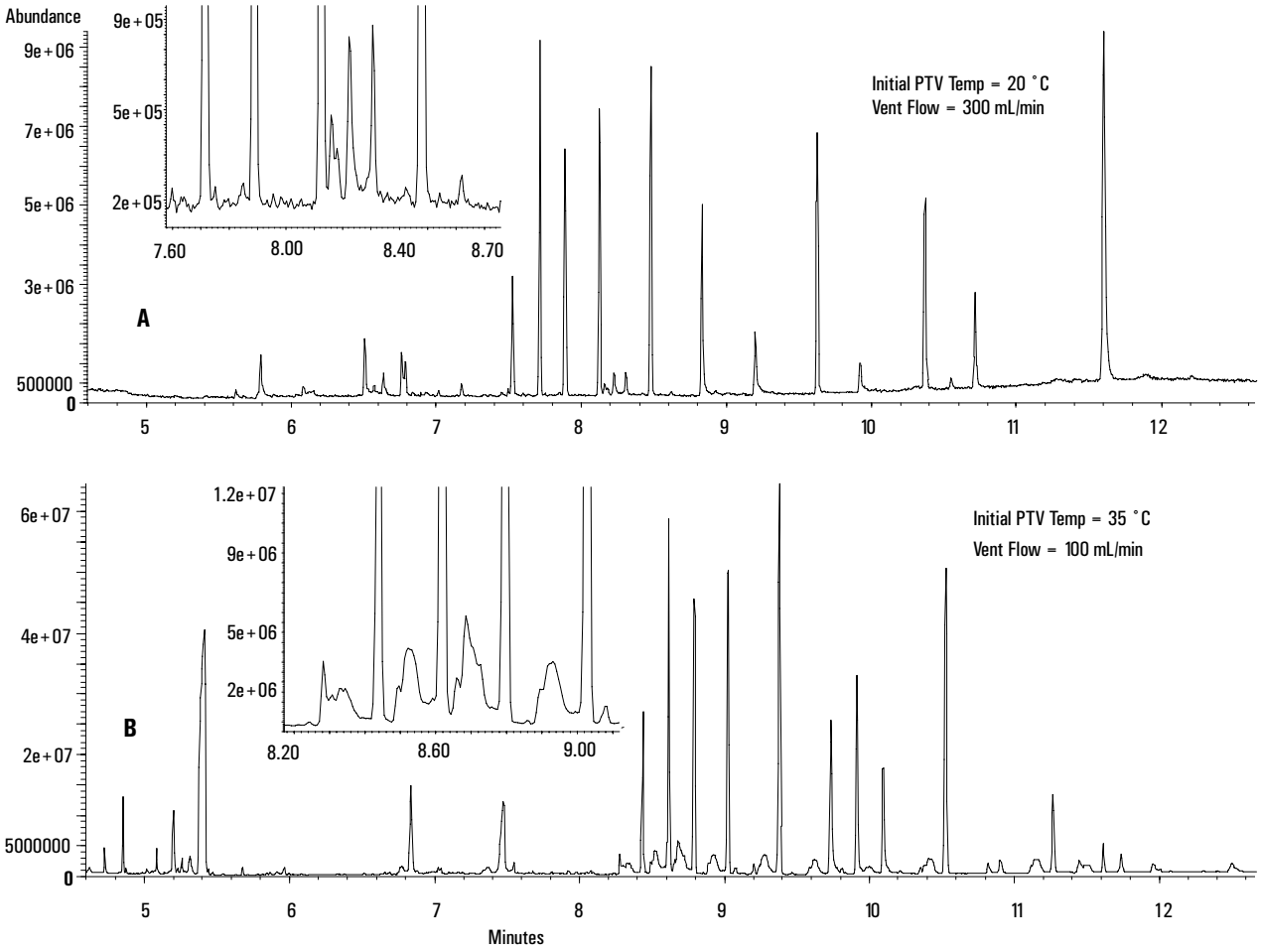



**Figure 5. Effect of PTV setpoints on peak shape**
**sample: pesticides at 1.0 ppm (peaks identified in figure 3); column: 30 m x 0.25 mm x 0.25 mm HP-5MS**

Agilent shall not be liable for errors contained herein or for incidental or consequential damages in connection with the furnishing, performance, or use of this material.

Information, descriptions, and specifications in this publication are subject to change without notice.

Copyright © 2000
Agilent Technologies, Inc.

Printed in the USA 2/2000
5965-7770E

**Agilent Technologies**
Innovating the HP Way